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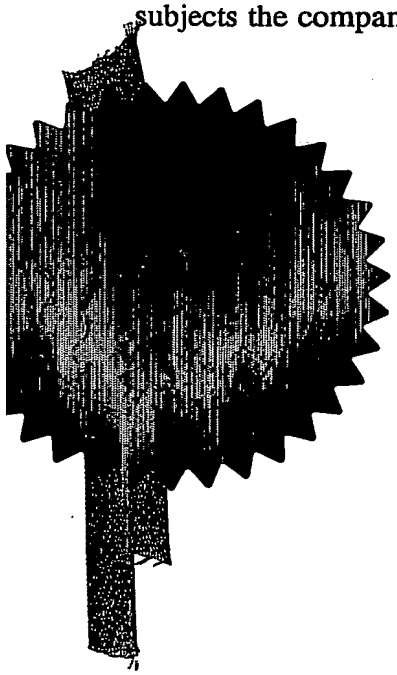

Signed Stephen Hordley

Dated 21 September 2004

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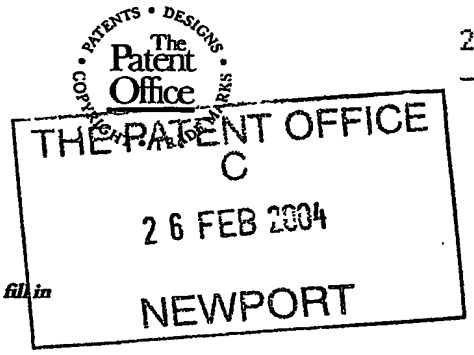

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Request for grant of a patent

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The Patent Office

Cardiff Road
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NP10 8QQ

1. Your reference — P104185GB

2. Patent application number *(The Patent Office will fill this part in)* — 0404242.0 — 26 FEB 2004

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Ludwig Institute for Cancer Research
Postfach 8024
Zurich
Switzerland

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — CH — 5771550002

4. Title of the invention — Screening Assay and Treatment

5. Name of your agent *(if you have one)*

Harrison Goddard Foote

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

31 St Saviourgate
York
YO1 8NQ
GB

Patents ADP number *(if you know it)* — 14571001 — 7914237002

6. Priority: Complete this section if you are declaring priority from one or more earlier patent applications, filed in the last 12 months.

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. Divisionals, etc: Complete this section only if this application is a divisional application or resulted from an entitlement dispute (see note f)

Number of earlier UK application	Date of filing *(day / month / year)*

8. Is a Patents Form 7/77 (Statement of inventorship and of right to grant of a patent) required in support of this request?

Answer YES if:

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.

Otherwise answer NO (See note d)

Yes

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9. Accompanying documents: A patent application must include a description of the invention. Not counting duplicates, please enter the number of pages of each item accompanying this form:

Continuation sheets of this form	
Description	30
Claim(s)	9
Abstract	1
Drawing(s)	33 + 33 RM.

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent (Patents Form 7/77)	
Request for a preliminary examination and search (Patents Form 9/77)	
Request for a substantive examination (Patents Form 10/77)	
Any other documents (please specify)	

11. I/We request the grant of a patent on the basis of this application.

Signature(s) [redacted] Date 25.2.04

12. Name, daytime telephone number and e-mail address, if any, of person to contact in the United Kingdom

Rob Dochertv

01904 732120

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b) Write your answers in capital letters using black ink or you may type them.

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Screening Assay and Treatment

The invention relates to an screening assay for the identification of agents which modulate the activity of polypeptides which affect the apoptotic activity of the tumour suppressor protein p53 and including gene therapy vectors comprising p53.

Tumour suppressor genes encode proteins which function to inhibit cell growth or division and are therefore important with respect to maintaining proliferation, growth and differentiation of normal cells. Mutations in tumour suppressor genes result in abnormal cell-cycle progression whereby the normal cell-cycle check points which arrest the cell-cycle, when, for example, DNA is damaged, are ignored and damaged cells divide uncontrollably. The products of tumour suppressor genes function in all parts of the cell (e.g. cell surface, cytoplasm, nucleus) to prevent the passage of damaged cells through the cell- cycle (i.e. G1, S, G2, M and cytokinesis). Arguably the tumour suppressor gene which has been the subject of the most intense research is p53. p53 encodes a protein which functions as a transcription factor and is a key regulator of the cell division cycle. It was discovered as a protein shown to bind with affinity to the SV40 large T antigen. The p53 gene encodes a 393 amino acid polypeptide with a molecular weight of 53kDa.

We have described a family of proteins in WO02/12325 which function to enhance the apoptotic activity of p53. ASPP1 and ASPP2 selectively interact with p53 to enhance the apoptotic function of p53 at p53 responsive promoters to promote apoptosis *in vivo*. We herein describe the interaction of ASPP family members with the oncogene Ras. ASPP 1 and 2 are also phophoproteins.

Ras oncogenes are frequently activated by mutation or over expression in many human tumours. For example, approximately 95% of pancreatic tumours contain so called K-Ras mutations. Ras oncogenes are believed to exert their effect by over-riding the normal cell-cycle control mechanisms by activating protein kinases (e.g. Raf, Mek, Erk kinase pathways) which regulate the function of cell-cycle cyclins which promote the proliferation of eukaryotic cells.

In its inactive state Ras is bound to GDP. The activation of Ras by growth factors results in exchange of GDP for GTP and a consequent change in the conformation of Ras to an activated form. In vitro, Ras has an intrinsic GTPase activity which becomes active when growth factor stimulation is removed and returns Ras to its GDP bound state. Ras is also a post-translationally modified protein and it is this modification which facilitates the localisation of Ras to the cell membrane and allows Ras to receive growth factor signals. The post-translational modification is farnesylation which results in the alkylation of cysteine residues in a conserved motif "CAAX". Ras has three CAAX motifs located in the C-terminus of the protein and it has been shown that inhibition of the farnesylation reaction of Ras blocks its processing and thereby inactivates the protein. The farnesylation reaction has been a target for the rational design of agents which inhibit the reaction thereby preventing the localisation of Ras at its site of action, the inner cell membrane. However, the farnesylation reaction is more complicated that was first seemed. For example, H Ras is exclusively modified by farnsyltransferase whereas K-Ras and N-Ras can also be modified by gerangylgerangyltransferase. The has meant that there is a continuing need to identify new targets which can modulate Ras activity or oncogenic Ras activity, either directly or indirectly,

We have identified ASPP 1/2 as Ras binding targets. The binding domain in ASPP1/2 is the amino terminus of the protein. We also show that Ras activates ASPP through the MAPK and Raf CX pathway and that dephosphorylation by phosphatase action is an important factor regulating ASPP action. The interaction of these protein factors with ASPP provides an opportunity to identify agents which enhance or inhibit the action of ASPP on p53 mediated apoptosis.

According to an aspect of the invention there is provided method for the identification of agents which modulate the interaction of the proto-oncogene/oncogene Ras with the p53 binding protein family ASPP, either directly or indirectly.

According to an aspect of the invention there is provided a screening method for the identification of agents which modulate the interaction of a first polypeptide encoded by a nucleic acid molecule selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b); with a second polypeptide selected from the group consisting of:

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 18a, 18c, 18e or 18g;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has the activity associated with Ras, or a variant Ras polypeptide;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e); comprising,

i) forming a preparation comprising said first and second polypeptide;

ii) adding at least one candidate agent to be tested; and

iii) determining the effect, or not, of said agent on the interaction of said first polypeptide with said second polypeptide.

In a preferred method of the invention said first polypeptide is represented by the amino acid sequence as shown in Figure 17c or 17d, or a variant polypeptide wherein said variant polypeptide sequence has been altered by addition, substitution or deletion of at least one amino acid residue.

A variant polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, truncations which may be present in any combination. Among preferred variants are those that vary from a reference polypeptide by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. The following non-limiting list of amino acids are considered conservative replacements (similar): a) alanine, serine, and threonine; b) glutamic acid and asparatic acid; c) asparagine and glutamine d) arginine and lysine; e) isoleucine, leucine, methionine and valine and f) phenylalanine, tyrosine and tryptophan.

In addition, the invention features polypeptide sequences having at least 75% identity with the polypeptide sequence as hereindisclosed, or fragments and functionally equivalent polypeptides thereof. In one embodiment, the polypeptides have at least 85% identity, more preferably at least 90% identity, even more preferably at least 95% identity, still more preferably at least 97% identity, and most preferably at least 99% identity with the amino acid sequence illustrated herein.

In a further preferred method of the invention said first polypeptide comprises the amino acid sequence +1 to +120 of the sequence shown in Fig 17c and 17d Preferably said polypeptide consists of the amino acid sequence +1 to +120 of the sequence shown in Figure 17c or 17d.

In a further preferred method of the invention said second polypeptide is represented by the amino acid sequence shown in Figure 18b, 18d, 18f or 18h, or a variant polypeptide wherein said variant polypeptide sequence has been altered by addition, substitution or deletion of at least one amino acid residue.

In a preferred method of the invention said second polypeptide is modified at amino acid residue 12. Preferably said modification is the substitution of amino acid 12 for amino acid valine. Preferably said second polypeptide is K-RasV12. Alternatively said polypeptide is H-RasV12.

In a yet further preferred method of the invention said second polypeptide is modified at amino acid residue 17. Preferably said modification is substitution of serine for asparagine at amino acid residue 17.

In a further preferred method of the invention said first and second polypeptides are expressed by a cell.

In a preferred method of the invention said cell is a cell transfected with at least one nucleic acid molecule(s) which encodes said first and second polypeptides.

Preferably the expression of said nucleic acid molecule(s) is regulatable.

In a preferred method of the invention said cell is a cancer cell.

In a yet further preferred method of the invention said cell is part of a transgenic animal wherein the genome of said animal has been modified to include nucleic acid molecules which encode first and second polypeptides. Preferably said nucleic acid molecules are expressed in a specific cell/tissue.

In a yet still further preferred method of the invention said preparation includes at least one chemotherapeutic agent.

According to an aspect of the invention there is provided a screening method for the identification of agents which modulate the phosphorylation of a first polypeptide encoded by a nucleic acid molecule selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b); with a second polypeptide selected from the group consisting of:

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 19 or 20;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has protein kinase activity;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e); comprising,

i) forming a preparation comprising said first and second polypeptide;

ii) adding at least one candidate agent to be tested; and

iii) determining the effect, or not, of said agent on the phosphorylation state of said first polypeptide.

According to a further aspect of the invention there is provided a screening method for the identification of agents which modulate the phosphorylation state of a first polypeptide encoded by a nucleic acid molecule selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b); with a second polypeptide selected from the group consisting of:

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 21;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has protein phosphatase activity;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e); comprising,

i) forming a preparation comprising said first and second polypeptide;

ii) adding at least one candidate agent to be tested; and

iii) determining the effect, or not, of said agent on the phosphorylation state of said first polypeptide.

In a preferred method of the invention said agent is a polypeptide.

In a preferred method of the invention said polypeptide is an antibody, or active binding fragment thereof.

Preferably said antibody or binding fragment is a monoclonal antibody.

Antibodies or immunoglobulins (Ig) are a class of structurally related proteins consisting of two pairs of polypeptide chains, one pair of light (L) (low molecular weight) chain (κ or λ), and one pair of heavy (H) chains (γ, α, μ, δ and ε), all four linked together by disulphide bonds. Both H and L chains have regions that contribute to the binding of antigen and that are highly variable from one Ig molecule to another. In addition, H and L chains contain regions that are non-variable or constant. The L chains consist of two domains. The carboxy-terminal domain is essentially identical among L chains of a given type and is referred to as the "constant" (C) region. The amino terminal domain varies from L chain to L chain and contributes to the binding site of the antibody. Because of its variability, it is referred to as the "variable" (V) region. The variable region contains complementarity determining regions or CDR's which form an antigen binding pocket. The binding pockets comprise H and L variable regions which contribute to antigen recognition. It is possible to create single variable regions, so called single

chain antibody variable region fragments (scFv's). If a hybridoma exists for a specific monoclonal antibody it is well within the knowledge of the skilled person to isolate scFv's from mRNA extracted from said hybridoma via RT PCR. Alternatively, phage display screening can be undertaken to identify clones expressing scFv's.

Alternatively said fragments are "domain antibody fragments". Domain antibodies are the smallest binding part of an antibody (approximately 13kDa). Examples of this technology is disclosed in US6, 248, 516, US6, 291, 158, US6,127, 197 and EP0368684 which are all incorporated by reference in their entirety.

In a preferred method of the invention said antibody fragment is a single chain antibody variable region fragment.

In a further preferred embodiment of the invention said antibody is a humanised or chimeric antibody.

A chimeric antibody is produced by recombinant methods to contain the variable region of an antibody with an invariant or constant region of a human antibody. A humanised antibody is produced by recombinant methods to combine the complementarity determining regions (CDRs) of an antibody with both the constant (C) regions and the framework regions from the variable (V) regions of a human antibody.

Chimeric antibodies are recombinant antibodies in which all of the V-regions of a mouse or rat antibody are combined with human antibody C-regions. Humanised antibodies are recombinant hybrid antibodies which fuse the complimentarity determining regions from a rodent antibody V-region with the framework regions from the human antibody V-regions. The C-regions from the human antibody are also used. The complimentarity determining regions (CDRs) are the regions within the N-terminal domain of both the heavy and light chain of the antibody to where the majority of the variation of the V-region is restricted. These regions form loops at the

surface of the antibody molecule. These loops provide the binding surface between the antibody and antigen.

Antibodies from non-human animals provoke an immune response to the foreign antibody and its removal from the circulation. Both chimeric and humanised antibodies have reduced antigenicity when injected to a human subject because there is a reduced amount of rodent (i.e. foreign) antibody within the recombinant hybrid antibody, while the human antibody regions do not elicit an immune response. This results in a weaker immune response and a decrease in the clearance of the antibody. This is clearly desirable when using therapeutic antibodies in the treatment of human diseases. Humanised antibodies are designed to have less "foreign" antibody regions and are therefore thought to be less immunogenic than chimeric antibodies.

In a further preferred method of the invention said agent is a peptide, preferably a modified peptide.

It will be apparent to one skilled in the art that modification to the amino acid sequence of peptides which modulate the interaction of ASPP family members with polypeptides involved in regulating ASPP activity could enhance the binding and/or stability of the peptide with respect to its target sequence. In addition, modification of the peptide may also increase the *in vivo* stability of the peptide thereby reducing the effective amount of peptide necessary to inhibit an interaction. This would advantageously reduce undesirable side effects which may result *in vivo*. Modifications include, by example and not by way of limitation, acetylation and amidation.

In a preferred method of the invention said peptide is acetylated. Preferably said acetylation is to the amino terminus of said peptide.

In a further preferred method of the invention said peptide is amidated. Preferably said amidation is to the carboxyl-terminus of said peptide.

In a further preferred method of the invention said peptide is modified by both acetylation and amidation.

Alternatively, or preferably, said modification includes the use of modified amino acids in the production of recombinant or synthetic forms of peptides. It will be apparent to one skilled in the art that modified amino acids include, by way of example and not by way of limitation, 4-hydroxyproline, 5-hydroxylysine, N^6-acetyllysine, N^6-methyllysine, N^6,N^6-dimethyllysine, N^6,N^6,N^6-trimethyllysine, cyclohexyalanine, D-amino acids, ornithine. Other modifications include amino acids with a C_2, C_3 or C_4 alkyl R group optionally substituted by 1, 2 or 3 substituents selected from halo (e.g. F, Br, I), hydroxy or C_1-C_4 alkoxy.

Alternatively, peptides could be modified by, for example, cyclisation. Cyclisation is known in the art, (see Scott et al Chem Biol (2001), 8:801-815; Gellerman et al J. Peptide Res (2001), 57: 277-291; Dutta et al J. Peptide Res (2000), 8: 398-412; Ngoka and Gross J Amer Soc Mass Spec (1999), 10:360-363.

In a preferred method of the invention peptides according to the invention are modified by cyclisation.

In a further preferred method of the invention said agent is an aptamer.

Nucleic acids have both linear sequence structure and a three dimensional structure which in part is determined by the linear sequence and also the environment in which these molecules are located. Conventional therapeutic molecules are small molecules, for example, peptides, polypeptides, or antibodies, which bind target molecules to produce an agonistic or antagonistic effects. It has become apparent that nucleic acid molecules also have potential with respect to providing agents with the requisite binding properties which may have therapeutic utility. These nucleic acid molecules are typically referred to as aptamers. Aptamers are small, usually

stabilised, nucleic acid molecules, which comprise a binding domain for a target molecule. A screening method to identify aptamers is described in US 5,270,163 which is incorporated by reference. Aptamers are typically oligonucleotides which may be single stranded oligodeoxynucleotides, oligoribonucleotides, or modified oligodeoxynucleotide or oligoribonucleotides.

The term "modified" encompasses nucleotides with a covalently modified base and/or sugar. For example, modified nucleotides include nucleotides having sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified nucleotides may also include 2' substituted sugars such as 2'-O-methyl-; 2-O-alkyl; 2-O-allyl; 2'-S-alkyl; 2'-S-allyl; 2'- fluoro-; 2'-halo or 2;azido-ribose, carbocyclic sugar analogues a-anomeric sugars; epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, and sedoheptulose.

Modified nucleotides are known in the art and include, by example and not by way of limitation, alkylated purines and/or pyrimidines; acylated purines and/or pyrimidines; or other heterocycles. These classes of pyrimidines and purines are known in the art and include, pseudoisocytosine; N4, N4-ethanocytosine; 8-hydroxy-N6-methyladenine; 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil; 5-fluorouracil; 5-bromouracil; 5-carboxymethylaminomethyl-2-thiouracil; 5-carboxymethylaminomethyl uracil; dihydrouracil; inosine; N6-isopentyl-adenine; 1-methyladenine; 1-methylpseudouracil; 1-methylguanine; 2,2-dimethylguanine; 2-methyladenine; 2-methylguanine; 3-methylcytosine; 5-methylcytosine; N6-methyladenine; 7-methylguanine; 5- methylaminomethyl uracil; 5-methoxy amino methyl-2-thiouracil; β-D-mannosylqueosine; 5-methoxycarbonylmethyluracil; 5-methoxyuracil; 2 methylthio-N6-isopentenyladenine; uracil-5-oxyacetic acid methyl ester; psueouracil; 2-thiocytosine; 5-methyl-2 thiouracil, 2-thiouracil; 4-thiouracil; 5-methyluracil; N-uracil-5-oxyacetic acid methylester; uracil 5—oxyacetic acid; queosine; 2-thiocytosine; 5-propyluracil; 5-propylcytosine; 5-ethyluracil; 5-

ethylcytosine; 5-butyluracil; 5-pentyluracil; 5-pentylcytosine; and 2,6,-diaminopurine; methylpsuedouracil; 1-methylguanine; 1-methylcytosine.

The aptamers of the invention are synthesised using conventional phosphodiester linked nucleotides and synthesised using standard solid or solution phase synthesis techniques which are known in the art. Linkages between nucleotides may use alternative linking molecules. For example, linking groups of the formula P(O)S, (thioate); P(S)S, (dithioate); P(O)NR'2; P(O)R'; P(O)OR6; CO; or CONR'2 wherein R is H (or a salt) or alkyl (1-12C) and R6 is alkyl (1-9C) is joined to adjacent nucleotides through –O- or –S-. The binding of aptamers to a target polypeptide is readily tested by assays hereindisclosed.

According to a further aspect of the invention there is provided a cell transfected with at least one nucleic acid molecule wherein the genome of said cell is modified to include at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b); and at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of:

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 18a, 18c, 18e, or 18g;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has the activity associated with Ras or a variant Ras polypeptide;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid

sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e) wherein said cell is adapted for the regulated expression of said nucleic acid molecules.

According to a further aspect of the invention there is provided a cell transfected with at least one nucleic acid molecule wherein the genome of said cell is modified to include at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b) and at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of;

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 19a or 20a;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has protein kinase activity;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e) wherein said cell is adapted for the regulated expression of said nucleic acid molecules.

According to a yet further aspect of the invention there is provided a cell transfected with at least one nucleic acid molecule wherein the genome of said cell is modified to include at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b) and at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of;

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 21a;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has protein kinase activity;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e) wherein said cell is adapted for the regulated expression of said nucleic acid molecules.

In a preferred embodiment of the invention said cell further comprises a nucleic acid molecule which includes a reporter gene to monitor the activity of said pro-apoptotic polypeptide(s).

In a preferred embodiment of the invention said cell is a cancer cell.

According to a yet further aspect of the invention there is provided a non-human transgenic animal comprising at least one cell according to the invention.

In a preferred embodiment of the invention said non-human animal is a non-human primate.

In a further preferred embodiment of the invention said transgenic animal is a rodent, preferably a mouse, rat or hamster.

In an alternative preferred embodiment of the invention said transgenic animal is a pig.

It is well within the knowledge of the skilled person to successfully generate both a heterozygous and homozygous transgenic animals with intergation of a desired nucleic acid encoding a particular gene or combination of genes using modified embryonic stem cells. These same steps can be easily applicable to species other than for example, mice. For example, Ware *et al* teaches an embryonic stem cell culture condition amenable for such animals as cattle, pigs and sheep (*Society for the Study of Reproduction*, 38:241 (1988)). In addition, this reference illustrates that the state of the art with respect to generation of transgenic species, other than mice, using modified embryonic stem cells is a well developed methodology.

According to an aspect of the invention there is provided a combined preparation of p53 and ASPP1 and/or ASPP2.

According to a further aspect of the invention there is provided a combined preparation comprising a nucleic acid molecule which encodes a p53 polypeptide, or sequence variant thereof, and at least one nucleic acid molecule which encodes at least one polypeptide, or sequence variant thereof, as represented by the amino acid sequences shown in Figure 17c and/or Figure 17d.

In a preferred embodiment of the invention there is provided a nucleic acid molecule which encodes both a p53 polypeptide and at least one polypeptide as represented by the amino acid sequences shown in Figure 17c and/or Figure 17d.

In a preferred embodiment of the invention said nucleic acid molecule is part of a vector. Preferably said nucleic acid molecules are operably linked to at least one promoter sequence which controls expression of said nucleic acid molecules.

"Promoter" is an art recognised term and, for the sake of clarity, includes the following features which are provided by example only. Enhancer elements are *cis* acting nucleic acid sequences often found 5' to the transcription initiation site of a gene (enhancers can also be found 3' to a gene sequence or even located in intronic sequences). Enhancers function to increase the rate of transcription of the gene to which the enhancer is linked. Enhancer activity is responsive to *trans* acting transcription factors which have been shown to bind specifically to enhancer elements.

Promoter elements also include so called TATA box and RNA polymerase initiation selection sequences which function to select a site of transcription initiation. These sequences also bind polypeptides which function, *inter alia,* to facilitate transcription initiation selection by RNA polymerase.

Adaptations also include the provision of selectable markers and autonomous replication sequences which facilitate the maintenance of said vector in either the eukaryotic cell or prokaryotic host. Vectors which are maintained autonomously are referred to as episomal vectors. Episomal vectors are desirable since these molecules can incorporate large DNA fragments (30-50kb DNA). Episomal vectors of this type are described in WO98/07876.

A number of viruses are commonly used as vectors for the delivery of exogenous genes. Commonly employed vectors include recombinantly modified enveloped or non-enveloped DNA and RNA viruses, preferably selected from baculoviridiae, parvoviridiae, picornoviridiae, herpesveridiae, poxviridae, adenoviridiae, or picornnaviridiae. Chimeric vectors may also be employed which exploit advantageous elements of each of the parent vector properties (See e.g., Feng, et al.(1997) Nature Biotechnology 15:866-870). Such viral vectors may be wild-type or may be modified by recombinant DNA techniques to be replication deficient, conditionally replicating or replication competent.

Preferred vectors are derived from the adenoviral, adeno-associated viral and retroviral genomes. In the most preferred practice of the invention, the vectors are

derived from the human adenovirus genome. Particularly preferred vectors are derived from the human adenovirus serotypes 2 or 5. The replicative capacity of such vectors may be attenuated (to the point of being considered "replication deficient") by modifications or deletions in the E1a and/or E1b coding regions. Other modifications to the viral genome to achieve particular expression characteristics or permit repeat administration or lower immune response are preferred. Most preferred are human adenoviral type 5 vectors containing a DNA sequence encoding the p53 tumor suppressor gene. In the most preferred practice of the invention as exemplified herein, the vector is replication deficient vector adenoviral vector encoding the p53 tumor suppressor gene A/C/N/53 as described in Gregory, et al., United States Patent No. 5,932,210 issued August 3, 1999 (the entire teaching of which is herein incorporated by reference).

Alternatively, the viral vectors may be conditionally replicating or replication competent. Conditionally replicating viral vectors are used to achieve selective expression in particular cell types while avoiding untoward broad spectrum infection. Examples of conditionally replicating vectors are described in Pennisi, E. (1996) Science 274:342-343; Russell, and S.J. (1994) Eur. J. of Cancer 30A(8):1165-1171. Additional examples of selectively replicating vectors include those vectors wherein an gene essential for replication of the virus is under control of a promoter which is active only in a particular cell type or cell state such that in the absence of expression of such gene, the virus will not replicate. Examples of such vectors are described in Henderson, et al., United States Patent No. 5,698,443 issued December 16, 1997 and Henderson, et al., United States Patent No. 5,871,726 issued February 16, 1999 the entire teachings of which are herein incorporated by reference.

Additionally, the viral genome may be modified to include inducible promoters which achieve replication or expression only under certain conditions. Examples of inducible promoters are known in the scientific literature (See, e.g. Yoshida and Hamada (1997) Biochem. Biophys. Res. Comm. 230:426-430; Iida, et al. (1996) J.

Virol. 70(9):6054-6059; Hwang, et al.(1997) J. Virol 71(9):7128-7131; Lee, et al. (1997) Mol. Cell. Biol. 17(9):5097-5105; and Dreher, et al.(1997) J. Biol. Chem 272(46); 29364-29371.

The viruses may also be designed to be selectively replicating viruses. Particularly preferred selectively replicating viruses are described in Ramachandra, et al. PCT International Publication No. WO 00/22137, International Application No. PCT/US99/21452 published April 20, 2000 and Howe, J., PCT International Publication No. WO WO0022136, International Application No. PCT/US99/21451 published April 20, 2000. A particularly preferred selectively replicating recombinant adenovirus is the virus dl01/07/309 as more fully described in Howe, J.

It has been demonstrated that viruses which are attenuated for replication are also useful in the therapeutic arena. For example the adenovirus dl1520 containing a specific deletion in the E1b55K gene (Barker and Berk (1987) Virology 156: 107) has been used with therapeutic effect in human beings. Such vectors are also described in McCormick (United States Patent No. 5,677,178 issued October 14, 1997) and McCormick, United States Patent No 5,846,945 issued December 8, 1998.

According to a further aspect of the invention there is provided a method to treat a condition which would benefit from an increase in apoptosis comprising administering a preparation comprising a first nucleic acid molecule comprising a nucleic acid sequence which encodes a p53 polypeptide, or sequence variant thereof, and administering a second preparation comprising a second nucleic acid molecule comprising a nucleic acid sequence which encodes a polypeptide, or sequence variant thereof, as represented by the amino acid sequence as shown in Figure 17c and/or Figure 17d wherein said preparations are administered simultaneously, sequentially or delayed manner.

According to a further aspect of the invention there is provided a method to treat a condition which would benefit from a stimulation of apoptosis comprising administering a combined preparation according to the invention.

In a preferred method of the invention said condition is cancer.

The preparations of the invention can be typically be administered orally, intravenously, subcutaneously, buccally, rectally, dermally, nasally, tracheally, bronchially, by any other parenteral route, as an oral or nasal spray or via inhalation.

An embodiment of the invention will now be described by example only and with reference to the following Figures:

Figure 1 illustrates the domains within ASPP2 which mediate ASPP2 activity;

Figure 2 illustrates that both H-ras V12 and K-ras V12 activate the ASPP2 and p53 synergy. Plasmids expressing CMV, p53, ASPP2 and ASPP2 + p53 were transfected in Saos-2 cells either with 1.5 μg of an empty vector, oncogenic H-ras or oncogenic K-ras. All samples were co-transfected with a bax-luciferase reporter. **A.** A transactivation assay was performed and shown here is the fold activation over p53. The average of duplicate samples are shown. **B, C** and **D.**Western blot of the samples used for transactivation. **B.** Expression of H-ras V12 and K-ras V12. **C.** Expression of ASPP2 and p53 for samples co-transfected with H-rasV12. **D.** Expression of ASPP2 and p53 for samples co-transfected with K-rasV12;

Figure 3 illustrates that average H-ras V12 and K-ras V12 activation of ASPP2 and p53 synergy;

Figure 4 illustrates Ras and Ras V12 activation of ASPP2 & p53 synergy on different promoters. Plasmids expressing CMV, p53, ASPP2 and ASPP2 + p53 were transfected either with 1 μg of an empty vector, wild type H-ras, dominant negative

H-ras N17 or oncogenic H-ras V12. All these samples were co-transfected with the indicated reporter. Luciferase activity was determined and the values are in relative luminescent units. The fold activation over p53 is shown. **A.** Bax-luciferase reporter, **B.** PIG3-luciferase reporter, **C.** Mdm2-luciferase reporter. **D.** The effect of H-rasV12 on ASPP2 and p53 activity on different reporters is shown. **E.** The fold activation of K-ras V12 and H-rasV12 over p53 and ASPP2 with the Bax-luciferase and the Mdm2-luciferase reporters.;

Figure 5 illustrates that oncogenic Ras activates endogenous ASPP1/2 and p53 to transactivate Bax-luciferase reporter. **A.** U2OS cells were transfected with 1 μg of either control vector or H-ras V12, each of which was also transfected with 6 μg of plasmids expressing either PCDNA or anti-sense ASPP2. All samples were co-transfected with bax-luciferase reporter. The lysates were then tested for luciferase levels. The values shown above are averages of duplicates. **B.** U2OS cells were transfected with 1 μg of a control vector pEF, H-ras V12 or K-ras V12, each of which were then co-transfected with 6 μg of plasmids expressing either PCDNA, anti-sense (α) ASPP1, αASPP2, or E6, or 4 μg of iASPP. All samples were co-transfected with Bax-luciferase. A transactivation assay was done on the lysates. The fold activation above the value of pEF and PCDNA control is shown. All samples were done in duplicate and the mean is shown here;

Figure 6A illustrates the testing of H-ras and K-ras RNAi constructs in pSUPPRESSOR and pSUPER vectors. Both HA-tagged H-rasV12 and HA-tagged K-rasV12 were co-transfected with either H-ras pSUPPRESSOR, K-ras pSUPPRESSOR, H-ras pSUPER or K-ras pSUPER. A western blot was performed with an HA antibody to see whether the different RNAi constructs were able inhibit expression in a specific manner. Effect of Ras RNAi on ASPP2 transactivation activity. Figure 6B illustrates H-ras pSUPER and K-ras pSUPER co-transfected with ASPP2 and/or p53 and its effect as monitored by the PIG3-luciferase counts.Figure 6C. A western blot was performed for each sample and blotted for ASPP2 and p53;

Figure 7 illustrates that activated Raf increases ASPP2 activity. **A.** p53 and ASPP2 were co-transfected with wild-type H-ras, dominant negative Ras N17, oncogenic H-ras V12 or Raf CX together with Bax-luciferase reporter. The values shown are the fold increase over p53 alone; Figure 7B and 7C illustrate activated Raf increases ASPP2 activity. **B.** p53 and ASPP2 were transfected with or without Raf CX. All samples were co-transfected with bax-luciferase. **C.** A western blot was performed with the samples shown in 7B and blotted against ASPP2, Raf and p53. Figure 7D illustrates that activated Raf increases ASPP2 activity. Raf CX increases p53 and ASPP2 synergy 2.5 fold (average of 3 experiments);

Figure 8 illustrates *in vitro* phosphorylation of the C-terminus of ASPP2. **A.** Small scale screening of ASPP2 phosphorylation by MAPK1, p70S6K, p90rsk, PKA, PKB and p83SAPK. The appropriate enzyme was added to a reaction containing P32 and either H2B as a positive control, ASPP2 or no substrate.**B.** A large scale *in vitro* phosphorylation assay was performed with ATP at higher P32 counts. PKA, p38SAPK, MAPK1 and p90rsk were used as kinases. The cpm counts of each band was measure and shown in **C.** **D.** The ASPP2 protein phosphorylated by MAPK was trypsinized and put through a High Performance Liquid Chromatography. The radioactive peptides fragments were collected and analysed by mass spectrometry. The second radioactive peak corresponds to a region of ASPP2 containing a putative MAPK phosphorylation site;

Figure 9 illustrates ASPP2 phosphorylation mutants. Three ASPP2 point mutant were made by replacing serines by alanine. Mutant 1 has a putative MAPK site mutated (PRPLSPT). Mutant 2 has the two serines at the putative PKA site mutated (TASSESP) and mutant 3 has the *in viro* phosphorylated MAPK site mutated (PAPSPG);

Figure 10 illustrates that RafCX is not able to transactivate ASPP2 mutant3. **A.**ASPP2 wild type and mutants were transfected in Saos2 cells with or without p53 and Bax-luciferase reporter activity measured. **B.** Bax-luciferase, ASPP2 wild type

and mutants were transfected with p53 and with or without 1.5 ug of RafCX. The values shown are the fold over ASPP2 and p53. This is the average of 3 experiments;

Figure 11 illustrates that endogenous ras binds the N-terminus of ASPP1. **A.** ASPP1 fragments that either contain or lack the ras-association domain (RAD). The column on the right shows the prediction for the different fragments to bind ras. **B.** U2OS cells were transfected with 10 ug of the different ASPP1 fragments. Lysates were made and endogenous ras immunoprecipitated with the 238 antibody. The immunoprecipitations were run on a gel and western blotted, probed with V5 to visualize the ASPP1 fragments. The left panel is the input and the right panel the immunoprecipitation;

Figure 12 illustrates that endogenous ras binds ASPP2 after EGF stimulation:. An inducible ASPP2 clone was used for an immunoprecipitation of endogenous ras. The cells were starved overnight, and then either stimulated with EGF or induced for ASPP2 expression or both simultaneously. The left panel shows ASPP2 and ras input whereas the right panel shows the immunoprecipitation of ras (238 antibody) and the immunoprecipitation with the control antibody (IgG);

Figure 13: illustrates that endogenous ASPP1 and ASPP2 bind endogenous activated ras. Saos2 cells were used for an immunoprecipitation of ASPP1 and ASPP2. The cells were starved overnight or grown in EGF and 20% fetal calf serum overnight. The western blot shows that Ras binds ASPP2 slightly more efficiently after EGF and 20%FCS stimulation. Endogenous ras is only co-immunoprecipitated by ASPP1 after the cells have been stimulated with EGF and FCS;

Figure 14 illustrates that purified N-term ASPP1 binds directly in a preferential manner to Ras.GTP. **A.** Purified ras was loaded with tritium labelled-GDP, -GTP or not loaded as a control. The cpm counts of the tritium are shown. The differences in cpm is due to the fact that GDP and GTP were not tritium-labelled at the same level. **B.** Relative loading of ras-GDP and ras-GTP, showing the mol of nucleotide bound

to ras. Both ras-GDP and ras-GTP were loaded with an equal amount of nucleotide.**C.** Loaded ras-GDP and ras-GTP with or without purified N-terminus ASPP1 was immunoprecipitated with a V5 antibody against the ASPP1 fragment. The immunoprecipitations were western blotted against ras to see whether there was direct and preferential binding of ras-GDP and ras-GTP to the amino-terminus of ASPP1. **D.** The intensity of the ras-GDP and ras-GTP bands pulled down by N-term ASPP1 were measured and shown as a graph. N-terminus ASPP1 binds ras-GTP four fold more strongly than it does to ras-GDP;

Figure 15 illustrates that ASPP2 co-localizes with H-ras V12. U2OS cells co-transfected with ASPP2 and H-rasV12 show co-localization of both proteins at the plasma membrane of the cells;

Figure 16 illustrates that K-rasV12 affects ASPP1 localization in a MAPK-dependent manner. After co-transfection of K-rasV12, ASPP1 changes its sub-cellular localization forming dense doughnut-like shapes. This change in localization is dependent on MAPK as a MAPK inhibitor UO126 reverts this change in localization;

Figure 17a is the ASPP1 nucleic acid sequence; Figure 17b is the ASPP2 nucleic acid sequence; Figure 17c is the ASPP1 protein sequence; Figure 17d is the ASPP2 protein sequence;

Figure 18a is the H-Ras wild-type nucleic acid sequence; Figure 18b is the H-Ras protein sequence; Figure 18c is the H-Ras oncogenic nucleic acid sequence; Figure 18d is the H-Ras oncogenic protein sequence; Figure 18e is the K Ras wild-type nucleic acid sequence; Figure 18f is the wild –type K-Ras protein sequence; Figure 18g is the K-Ras oncogenic nucleic acid sequence; and Figure 18h is the K-Ras oncogenic protein sequence;

Figure 19a is the MAPK nucleic acid sequence; Figure 19b is the MAPK protein sequence;

Figure 20a is the PKA nucleic acid sequence; Figure 20b is the PKA protein sequence;

Figure 21a is the phosphatase 1 nucleic acid sequence; Figure 21b is the phosphatase 1 protein sequence; and

Figure 22 illustrates the effect of H-ras RNAi and K-ras RNAi on apoptosis.

EXAMPLE 1

The amino terminus of ASPP1 and ASPP2 is necessary for its full activity

p53 is the most common tumour suppressor protein found mutated in cancers with more than half of all human cancers carrying p53 mutations. p53 is activated by stress signals such as DNA damage and its activation can lead to one of two responses: cell cycle arrest or apoptosis. It has recently been shown that a novel family of tumour suppressor proteins, known as the ASPP family, can interact with p53 and specifically enhance p53-induced apoptosis but not cell-cycle arrest. ASPP1 and ASPP2 enhance the DNA binding and transactivation function of p53 on the promoters of pro-apoptotic genes only in vivo. Mutant ASPP1 and ASPP2 missing the first 150 amino acids are not fully functional, suggesting that the domain is essential for full activity of the ASPP proteins. This amino-terminal domain that is required for ASPP activity contains a putative Ras-association domain (see Figure 1).

EXAMPLE 2

Oncogenic H-rasV12 and K-rasV12 increases ASPP2 transactivation activity

It has previously been reported that ASPP2 can increase p53 transactivation on pro-apoptotic genes and we wanted to see whether ras would have any effect on ASPP2 activity. We have shown, using transactivation assays, that both oncogenic H-rasV12 and K-rasV12 can increase ASPP2 activity 2-3 fold (see figure 2 and 3) on pro-apoptotic p53 reporters. Knowing that oncogenic RasV12 could activate ASPP2 activity, the roles of wild type Ras and its dominant negative version (Ras N17) were investigated. Interestingly, wild type H-ras seemed to have no effect on ASPP2 and p53 synergy whereas dominant negative ras reduced significantly the synergy, suggesting that endogenous ras activiy is needed for the full activity of ASPP2 on the bax-luciferase reporter (Figure 4A). To see whether the induction of ASPP activity by Ras is promoter specific, different p53 reporters were compared: both pro-apoptotic (such as Bax-luciferase and PIG3-luciferase) and non-apoptotic (Mdm2-luciferase). We can see that in all cases oncogenic ras activates ASPP2 2-3 fold irrespective of the promoter (figure 4A-C). Although there does not seem to be any promoter specificity regarding ras activation of ASPP (figure 4E), since ASPP2 has an intrinsic pro-apoptotic activity and only activates p53 transactivation on pro-apoptotic reporters, the overall effect of Ras is to significantly increase ASPP2 activity on pro-apoptotic reporters. (Figure 4D).

EXAMPLE 3

Oncogenic ras can activate endogenous ASPP1, ASPP2 and p53 to transactivate a pro-apoptotic reporter

U2OS cells were used to look at the effect of oncogenic ras on endogenous ASPP2 and p53. Figure 5A shows that H-ras V12 increases the bax-luciferase counts and that this inhibition is inhibited in the presence of anti-sense ASPP2, suggesting that the

activity is via endogenous ASPP2. Both ASPP1 and ASPP2 seem necessary for the pro-apoptotic activity of H-rasV12 and K-rasV12. Removal of endogenous ASPP1/2 by anti-sense DNA inhibits oncogenic ras activation to the same levels as adding iASPP (figure 5B). The addition of the p53-inhibitor E6 also reduces oncogenic ras activation of bax-luciferase significantly. These results suggest that oncogenic ras can activate bax-luciferase via endogenous ASPP1, ASPP2 and p53.

EXAMPLE 4

Endogenous H-ras is necessary for full ASPP2 activity

H-ras and K-ras RNAi constructs were made in both the pSUPPPRESSOR and pSUPER vectors. Only the constructs in the pSUPER vector were able to reduce ras levels specifically as shown in Figure 6A. The pSUPER constructs were therefore tested in a transactivation assay and H-ras RNAi was able to reduce ASPP2 and p53 transcriptional activity as shown in Figure 6B. All the constructs were expressed (Figure 6C) although there was no difference in ras levels after H-ras RNAi or K-ras RNAi (figure not shown); this is probably due to the very high endogenous levels of ras in those cells.

EXAMPLE 5

Ras activates ASPP2 via its Raf-MAPK pathway

Ras is a GTPase which is upstream of many effector pathways, the most well-known of which is the Raf-MAPK pathway. As it was unclear as to whether ras activated ASPP2 directly or via one of its pathways, we looked to see whether an activated form of Raf had any effect on ASPP2 activity. Figure 7 shows that Raf CAAX (an activated form of Raf) could increase ASPP2 and p53 synergy at least as much as H-rasV12, suggesting that oncogenic ras activates ASPP2 via its Raf pathway.

EXAMPLE 6

The C-term of ASPP2 is phosphorylated in vitro by MAPK and Raf CX is unable to activate a phosphorylation mutant of ASPP2

The purified C-terminus of ASPP2 was used as a substrate to screen for kinases in an in vitro assay. An array of kinases were added to the purified C-terminus of ASPP2 and the phosphorylation status of the protein was analysed using P32 as a phosphorylation marker. MAPK1, PKA, p38SAPK and p90rsk were all found to be able to phosphorylate ASPP2 (figure 8A). A larger scale in vitro phosphorylation assay was then performed, using the four enzymes that had screened positive in the first round (figure 8B-C). The phosphorylated fragment of ASPP2 was run on a gel, exposed, extracted from the gel and trypsinized. The trypsinized protein was then put through a High Performance Liquid Chromatography (HPLC) with an acetonitrile gradient, the radioactive peptides were then collected and analyzed by mass spectrometry (figure 8D). For the MAPK-phosphorylated ASPP2 C-terminus, there were two phosphorylation sites. The first one corresponded to the linker region between the GST and the protein. The second phosphorylated site corresponded to a region of the protein that contains a MAPK-consensus sequence phosphorylation site; PAPSPGL.

ASPP2 phosphorylation mutants were then constructed to see whether this site is phosphorylated in vivo. A serine was replaced by an alanine residue at the MAPK phosphorylation site identified by the in vitro phosphorylation assay (mutant 3), at another MAPK putative site (mutant 1) and at a PKA putative site that was shown to be phosphorylated in vitro (mutant 2) as shown in figure 9.

The ASPP2 mutants were compared to wild type ASPP2 in a transactivation assay with p53 using bax-luciferase as a reporter (figure 10A). The activity of all three ASPP2 mutants were equivalent to ASPP2 wild type activity. We then wanted to see if RafCX was able to increase ASPP2 wild type and mutants activity. p53 and ASPP2

wild type or mutants were transfected with and without RafCX and the bax-luciferase reporter activity measured. The values shown in Figure 10B show the fold of activation of RafCX over p53 and ASPP2 values. RafCX is shown to increase ASPP2 wild type, mutant 1 and mutant 2 activity but not ASPP2 mutant 3 activity. ASPP2 mutant three is mutated in the putative MAPK site that was shown to phosphorylate ASPP2 in vitro. This suggests that RafCX, by activating its downstream effector pathway MAPKK-MAPK, leads to MAPK phosphorylation of ASPP2 at the serine site PAPSPGL (amino acid 827) and that phosphorylation on this serine is necessary for RafCX activation of ASPP2.

EXAMPLE 8

H-ras binds to ASPP1 in its amino-terminus

As ASPP1 and ASPP2 contain a putative ras-association domain in their amino-terminus, we investigated whether ASPP was able to bind ras. We used different fragments of ASPP1 to see what parts of the protein bound to ras (figure 11A). Fragment 1 contains the N-terminus of the protein, fragment 2 lacks the C-terminus, KIA lacks part of the ras-association domain, fragment 6 lacks the entire ras-association domain and fragment 8 contains only the carboxy-terminus. Endogenous ras was immunoprecipitated and the fragments blotted for by western blot. As figure 11B shows, all fragments containing the putative ras-association domain (full length ASPP1, fragment 1 and fragment 2) were pulled down by endogenous ras. The fragments lacking the ras-association domain (fragment 6 and fragment 8) were unable to bind to endogenous ras. Fragment KIA which contained part of the ras association domain was immunoprecipitated by endogenous ras; this might be due to this fragment heterodimerizing with full-length, wild-type endogenous ASPP1 which contains the entire ras-association domain.

EXAMPLE 9

Activated endogenous ras binds ASPP1 and ASPP2

EGF ligand activates EGFR, which leads to the recruitment of a guanine nucleotide exchange factor (GNEF). Once recruited to the plasma membrane and in close proximity to Ras, the GNEF leads to the exchange of GDP- to GTP-associated ras, making ras active and able to activate its downstream effectors. Thus EGF is able to activate endogenous ras in a physiological manner. An ASPP2 inducible clone was used to see whether endogenous, wild-type ras bound ASPP2 with different activity after stimulation with EGF. An immunoprecipitation was performed with endogenous ras, with and without ASPP2 and ras induction. Figure 12 shows that there is very little background binding of endogenous ras and endogenous ASPP2 after cells have been starved for 24 hours. Inducing ASPP2 expression shows a small band of ASPP2 being co-immunoprecipitated by endogenous ras. Inducing endogenous ras by EGF and foetal calf serum leads to a significant increase in ras and ASPP2 binding.

An immunoprecipitation was performed to determine whether endogenous ras is able to bind to endogenous ASPP1 and ASPP2. Figure 13 shows that induced ras is co-immunoprecipitated with endogenous ASPP1, whereas non-induced ras was not able to bind to ASPP1. Although the difference in binding of ASPP2 to induced versus non-induced ras was not as clear, we can still see a slight increase of ASPP2 binding to induced ras compared to non-induced ras.

EXAMPLE 10

The amino-terminus of ASPP1 binds ras.GTP more efficiently than ras.GDP

In order to determine whether the interaction of ras and ASPP1/2 is direct, as we would expect if ASPP contains an active ras-association domain, we performed an in vitro binding assay with purified ras and amino-terminus of ASPP1. As suggested

with the EGF-dependent binding of ras to ASPP, we speculated that ras in its GTP form could bind the amino-terminus of ASPP1 more efficiently than ras in its GDP form. We therefore purified ras and loaded it with either GDP or GTP (figure 14A-B). The loaded ras was mixed with the purified amino-terminus-ASPP1 or without it as a control and the amino-terminus of ASPP1 was immunoprecipitated with a V5 antibody. As figure 14C-D shows, the N-terminus of ASPP1 binds ras-GTP with four-fold more efficiency than to ras-GDP.

EXAMPLE 11

Oncogenic ras co-localizes with ASPP2 but not ASPP1 and changes the ASPP1 cellular localization.

U2OS cells co-transfected with oncogenic H-rasV12 and ASPP2 clearly show co-localization at the plasma membrane of the cells (figure 15). However H-rasV12 and ASPP1 are not seen to co-localize. ASPP1, unlike ASPP2, is not found at the plasma membrane (figure 16). After co-transfection of K-rasV12, ASPP1 forms dense doughnut-like shapes in the middle of the cells. However, after addition of a MAPK-inhibitor UO126, ASPP1 resumes its "normal" cellular localization pattern.

Claims

1. A screening method for the identification of agents which modulate, either directly or indirectly, the interaction of a first polypeptide encoded by a nucleic acid molecule selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b); with a second polypeptide selected from the group consisting of:

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 18a, 18c, 18e or 18g;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has the activity associated with Ras or a variant Ras polypeptide;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e); comprising,

i) forming a preparation comprising said first and second polypeptide;

ii) adding at least one candidate agent to be tested; and

iii) determining the effect, or not, of said agent on the interaction of said first polypeptide with said second polypeptide.

2. A method according to Claim 1 wherein said first polypeptide is represented by the amino acid sequence as shown in Figure 17c or 17d, or a variant polypeptide wherein said variant polypeptide sequence has been altered by addition, substitution or deletion of at least one amino acid residue.

3. A method according to Claim 1 or 2 wherein said first polypeptide comprises the amino acid sequence + 1 to +120 of the sequence shown in Fig 17c and 17d .

4. A method according to Claim 3 wherein said polypeptide consists of the amino acid sequence +1 to +120 of the sequence shown in Figure 17c or 17d.

5. A method according to any of Claims 1-4 wherein said second polypeptide is represented by the amino acid sequence shown in Figure 18b, 18d, 18f or 18h, or a variant polypeptide wherein said variant polypeptide sequence has been altered by addition, substitution or deletion of at least one amino acid residue.

6 A method according to Claim 5 wherein said second polypeptide comprises the amino acid sequence as shown in Figure 18d.

7. A method according to Claim 5 or 6 wherein said second polypeptide comprises the amino acid sequence as shown in Figure 18h.

8. A method according to any of Claims 1-4 wherein said second polypeptide is modified at amino acid residue 17.

9. A method according to Claim 8 wherein said modification is the substitution of a serine amino acid for an asparagine amino acid.

10. A method according to any of Claims 1-9 wherein said first and second polypeptides are expressed by a cell.

11. A method according to Claim 10 wherein said cell is a cell transfected with at least one nucleic acid molecule(s) which encodes said first and second polypeptides.

12. A method according to Claim 10 or 11 wherein the expression of said nucleic acid molecule(s) is regulatable.

13. A method according to any of Claims 10-12 wherein said cell is a cancer cell.

14. A method according to any of Claims 10-13 wherein said cell is part of a transgenic animal wherein the genome of said animal has been modified to include nucleic acid molecules which encode first and second polypeptides.

15. A method according to any of Claims 10-14 wherein said nucleic acid molecules are expressed in a specific cell/tissue.

16. A screening method for the identification of agents which modulate, either directly or indirectly, the phosphorylation of a first polypeptide encoded by a nucleic acid molecule selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b); with a second polypeptide selected from the group consisting of:

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 19a or 20a;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has protein kinase activity;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e); comprising,

i) forming a preparation comprising said first and second polypeptide;

ii) adding at least one candidate agent to be tested; and

iii) determining the effect, or not, of said agent on the phosphorylation state of said first polypeptide.

17. A screening method for the identification of agents which modulate, either directly or indirectly, the phosphorylation state of a first polypeptide encoded by a nucleic acid molecule selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b); with a second polypeptide selected from the group consisting of:

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 21a;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has protein phosphatase activity;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e); comprising,

i) forming a preparation comprising said first and second polypeptide;

ii) adding at least one candidate agent to be tested; and

iii) determining the effect, or not, of said agent on the phosphorylation state of said first polypeptide.

18. A method according to any of Claims 1-17 wherein said agent is a polypeptide.

19. A method according to Claim 18 wherein said polypeptide is an antibody, or active binding fragment thereof.

20. A method according to Claim 19 wherein said antibody or binding fragment is a monoclonal antibody.

21. A method according to Claim 19 or 20 wherein said antibody fragment is a single chain antibody variable region fragment or a domain antibody fragment.

22. A method according to Claim 19 or 20 wherein said antibody is a humanised or chimeric antibody.

23. A method according to any of Claims 1-17 wherein said agent is a peptide.

24. A method according to any of Claims 1-17 wherein said agent is an aptamer.

25. A cell transfected with at least one nucleic acid molecule wherein the genome of said cell is modified to include at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b); and at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of:

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 18a, 18c, 18e or 18g;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the

nucleic acid molecule in (d) above and which has the activity associated with Ras or a variant Ras polypeptide;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e) wherein said cell is adapted for the regulated expression of said nucleic acid molecule(s).

26. A cell transfected with at least one nucleic acid molecule wherein the genome of said cell is modified to include at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b) and at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of;

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 19a or 20a;

e) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has protein kinase activity;

f) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e) wherein said cell is adapted for the regulated expression of said nucleic acid molecule(s).

27. A cell transfected with at least one nucleic acid molecule wherein the genome of said cell is modified to include at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of:

a) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid

sequence as represented in Figure 17a or 17b;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (a) and which enhances the pro-apoptotic activity of p53;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (a) and (b) and at least one copy of a nucleic acid molecule encoding a polypeptide selected from the group consisting of;

d) a polypeptide encoded by a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 21a;

b) a polypeptide encoded by a nucleic acid molecule which hybridises to the nucleic acid molecule in (d) above and which has protein phosphatase activity;

c) a polypeptide encoded by a nucleic acid molecule consisting of a nucleic acid sequence that is degenerate as a result of the genetic code to a nucleic acid molecule as defined in (d) and (e) wherein said cell is adapted for the regulated expression of said nucleic acid molecule(s).

28. A cell according to any of Claims 25-27 wherein said cell further comprises a nucleic acid molecule which includes a reporter gene to monitor the activity of said pro-apoptotic polypeptide(s).

29. A cell according to any of Claims 25-28 wherein said cell is a cancer cell.

30. A non-human transgenic animal comprising at least one cell according to any of Claims 25-29.

31. An animal according to Claim 30 wherein said non-human animal is a non-human primate.

32. An animal according to Claim 30 wherein said transgenic animal is a rodent.

33. An animal according to Claim 30 wherein said transgenic animal is a pig.

34. A combined preparation of p53 and ASPP1 and/or ASPP2.

35. A combined preparation comprising a nucleic acid molecule which encodes a p53 polypeptide, or sequence variant thereof, and at least one nucleic acid molecule which encodes at least one polypeptide, or sequence variant thereof, as represented by the amino acid sequences shown in Figure 17c and/or Figure 17d.

36. A preparation according to Claim 35 wherein both a p53 polypeptide and at least one polypeptide as represented by the amino acid sequences shown in Figure 17c and/or Figure 17d is encoded by a single nucleic acid molecule.

37. A preparation according to Claim 35 or 36 wherein said nucleic acid molecule is part of a vector.

38. A preparation according to Claim 37 wherein said nucleic acid molecules are operably linked to at least one promoter sequence which controls expression of said nucleic acid molecules.

39. A method to treat a condition which would benefit from an increase in apoptosis comprising administering a preparation comprising a first nucleic acid molecule comprising a nucleic acid sequence which encodes a p53 polypeptide, or sequence variant thereof, and administering a second preparation comprising a second nucleic acid molecule comprising a nucleic acid sequence which encodes a polypeptide, or sequence variant thereof, as represented by the amino acid sequence as shown in Figure 17c and/or Figure 17d wherein said preparations are administered simultaneously, sequentially or delayed manner.

40. A method to treat a condition which would benefit from a stimulation of apoptosis comprising administering a combined preparation according to any of Claims 34-38.

41. A method according to Claim 39 or 40 wherein said condition is cancer.

Abstract

Screening Assay and Treatment

The invention relates to an screening assay for the identification of agents which modulate the activity of polypeptides which affect the apoptotic activity of the tumour suppressor protein p53 and including gene therapy vectors comprising p53.

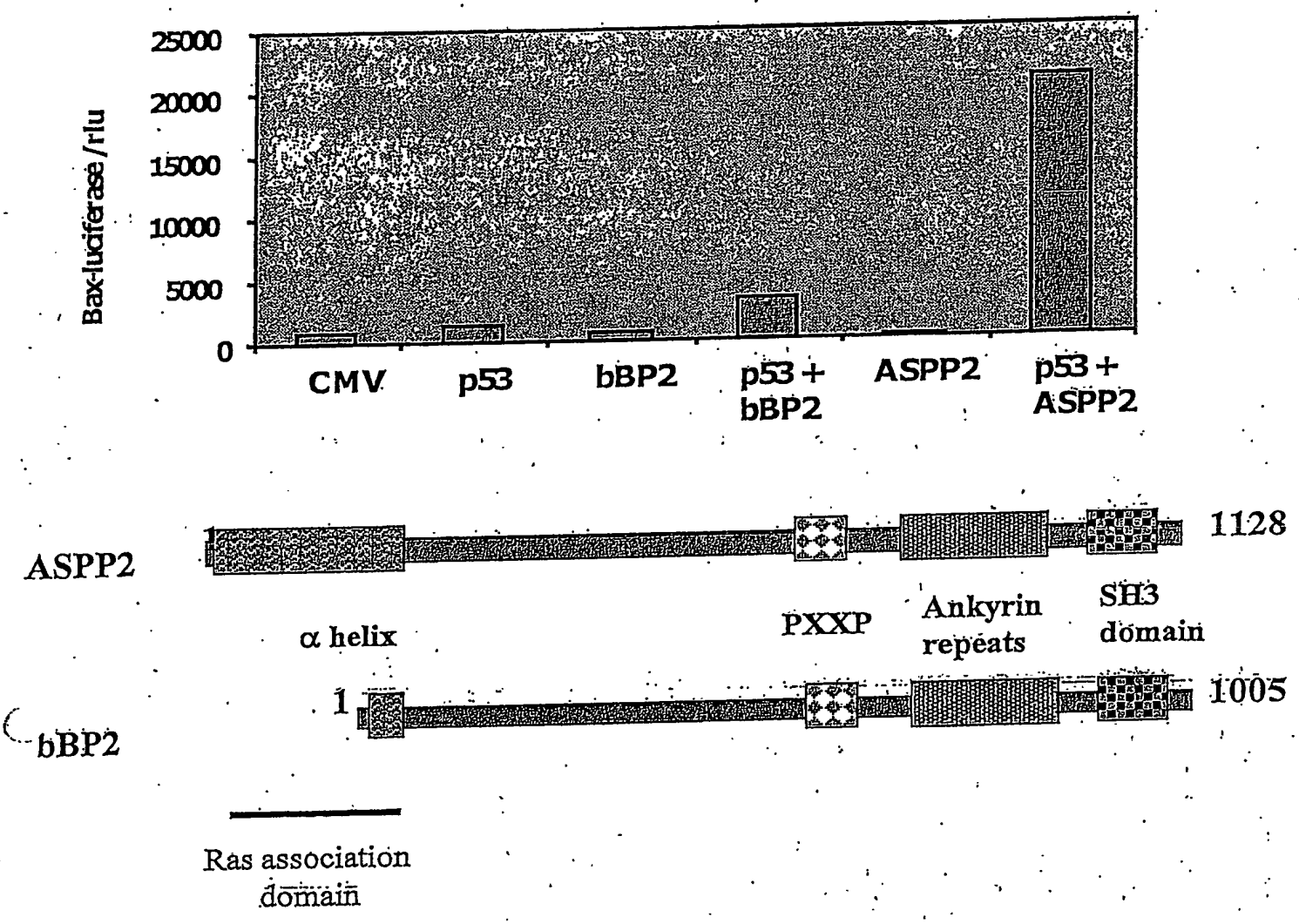
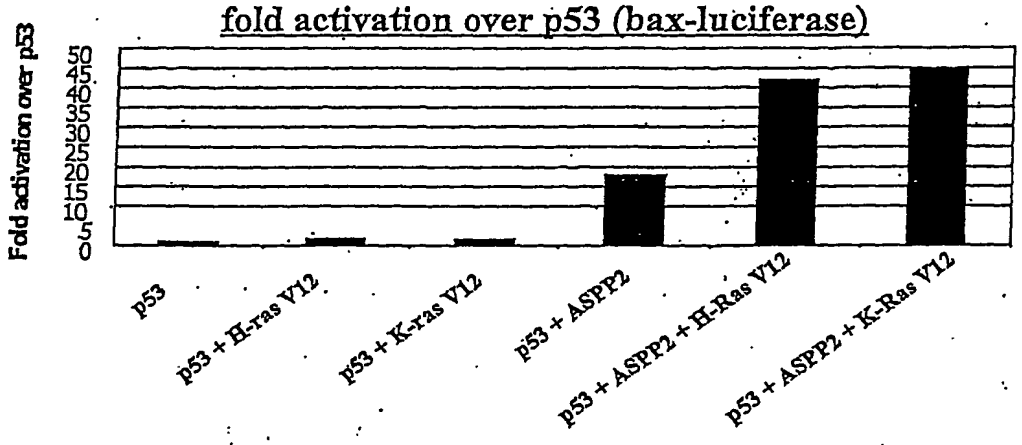
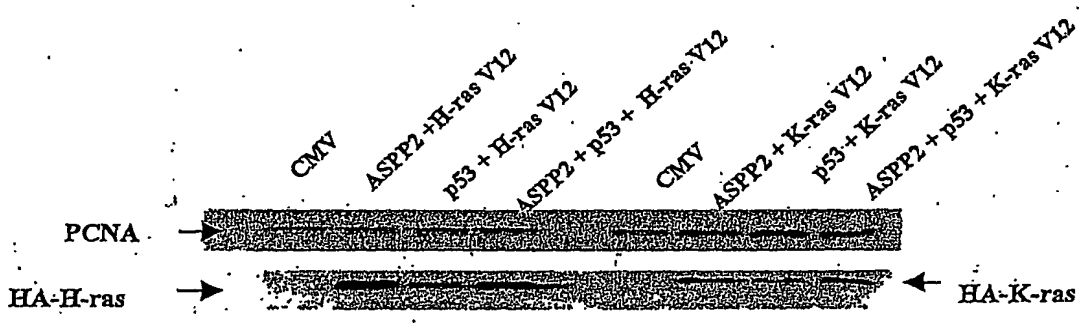
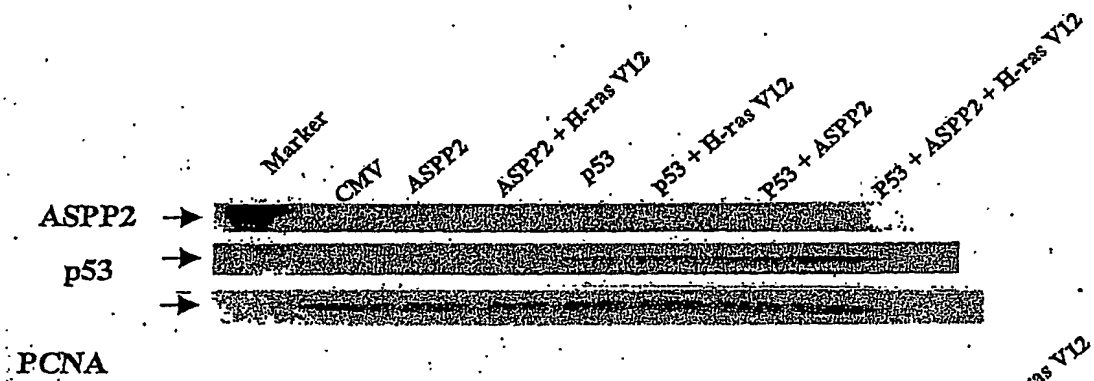


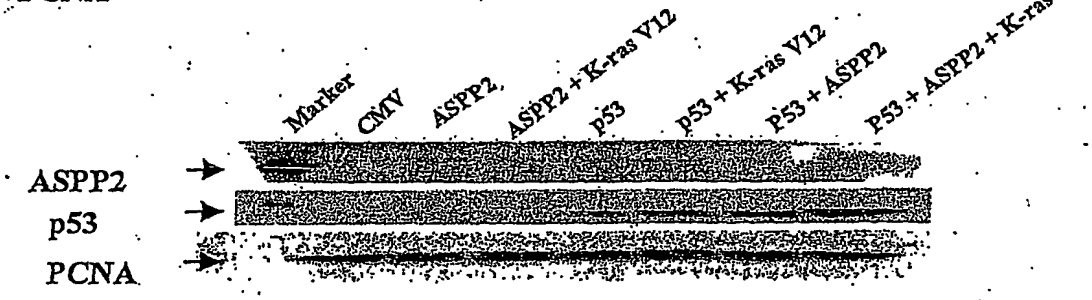
Figure 1: The N-terminal 130 amino acids of ASPP2 is crucial for its full activity

Fig 2


fold activation over p53 (bax-luciferase)
Fold activation over p53
0
5
10
15
20
25
30
35
40
45
50
p53
p53 + H-ras V12
p53 + K-ras V12
p53 + ASPP2
p53 + ASPP2 + H-Ras V12
p53 + ASPP2 + K-Ras V12


CMV
ASPP2 +H-ras V12
p53 + H-ras V12
ASPP2 + p53 + H-ras V12
CMV
ASPP2 + K-ras V12
p53 + K-ras V12
ASPP2 + p53 + K-ras V12
PCNA
HA-H-ras
HA-K-ras


Marker
CMV
ASPP2
ASPP2 + H-ras V12
p53
p53 + H-ras V12
P53 + ASPP2
P53 + ASPP2 + H-ras V12
ASPP2
p53
PCNA


Marker
CMV
ASPP2
ASPP2 + K-ras V12
p53
p53 + K-ras V12
P53 + ASPP2
P53 + ASPP2 + K-ras V12
ASPP2
p53
PCNA

H-ras and K-ras activate ASPP equally

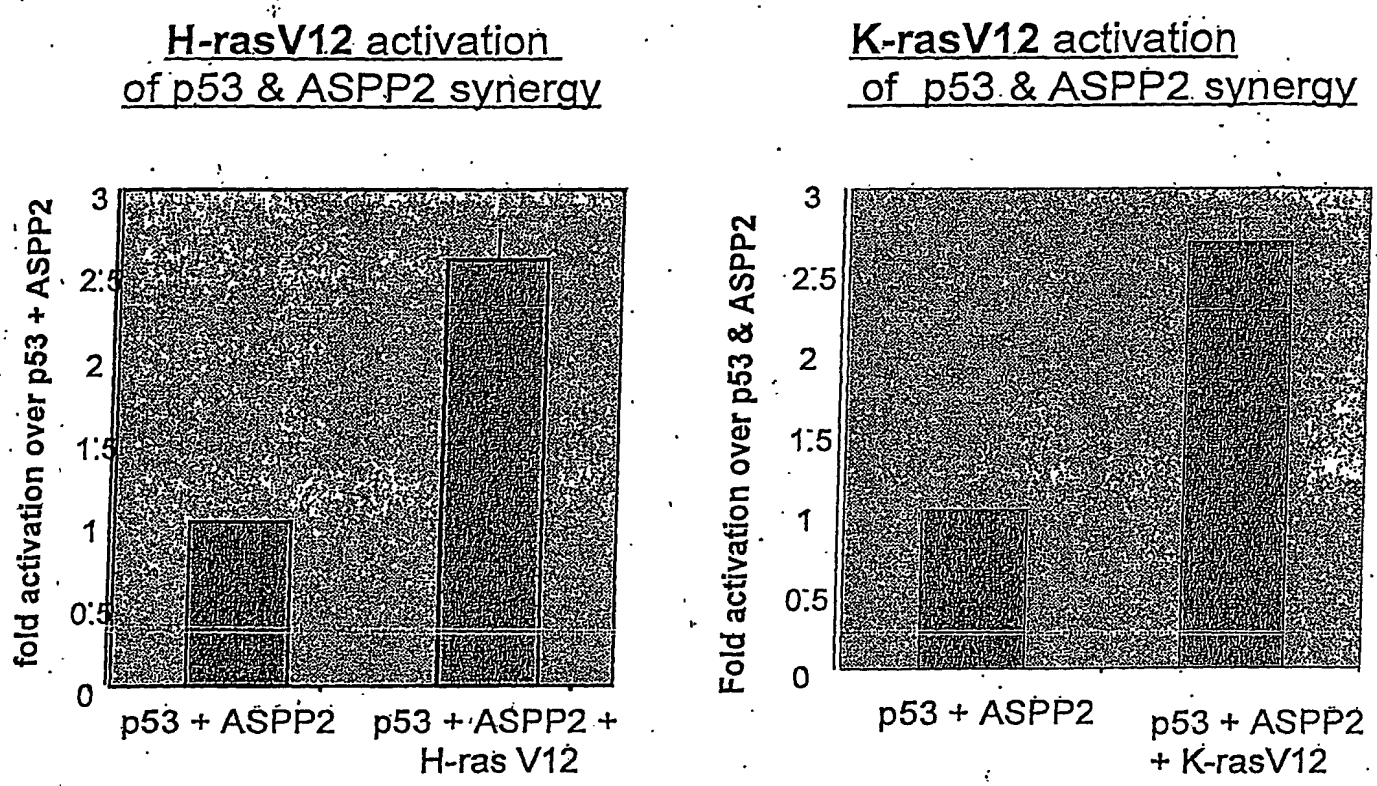


Figure 3:

Promoter specificity

Figure 4A

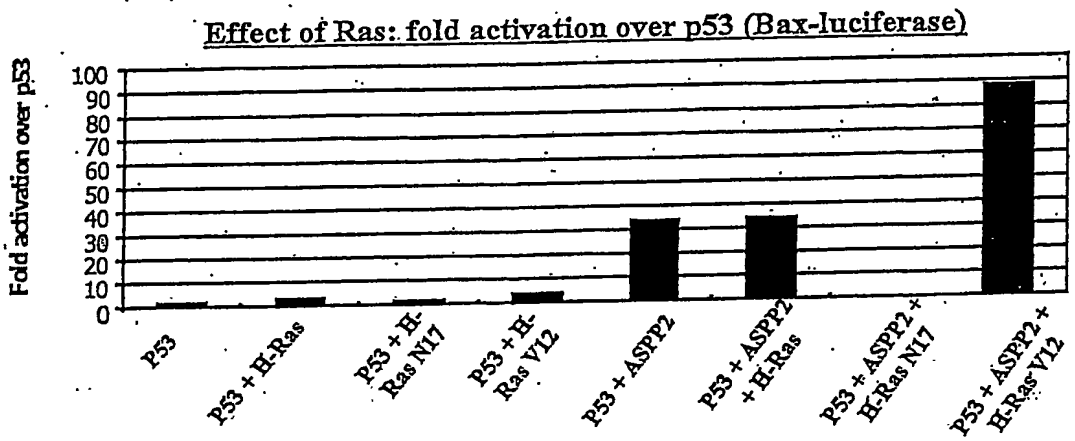


Figure 4B

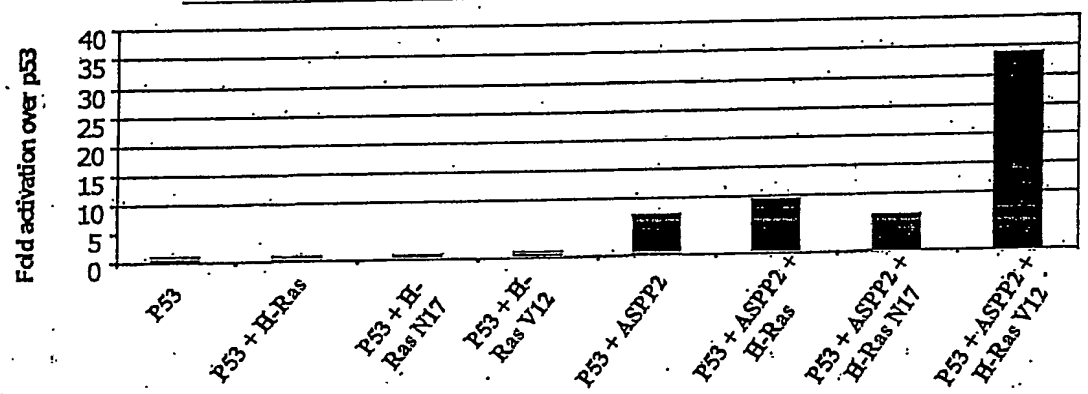


Figure 4C

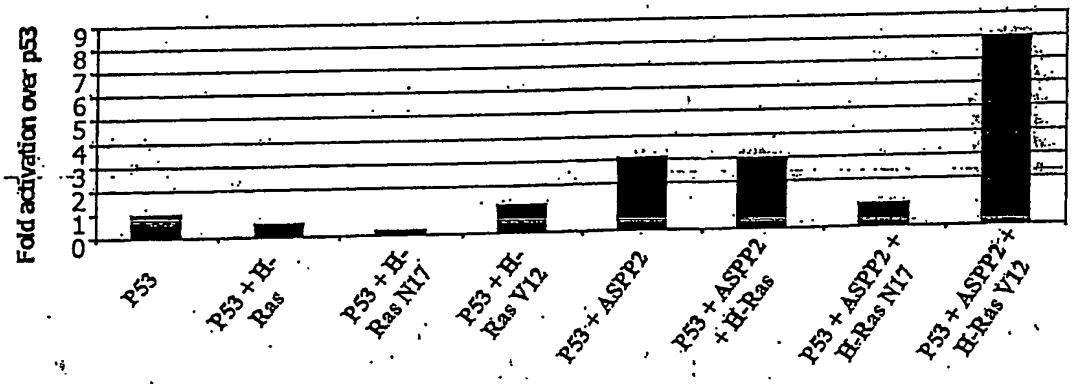


Figure 4D

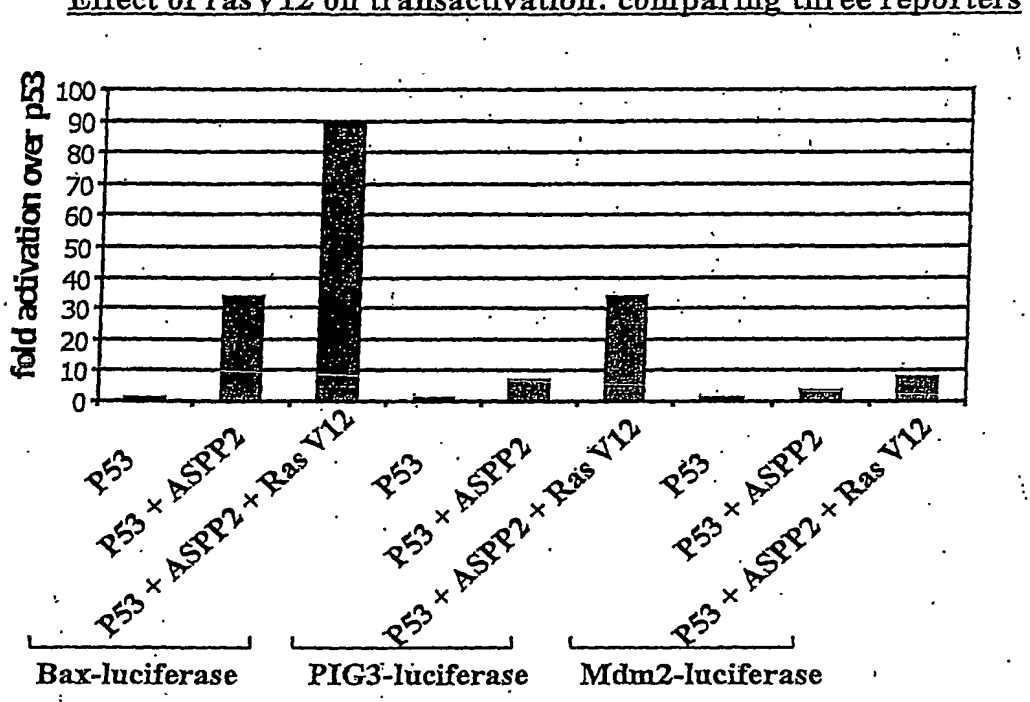


Figure 4E

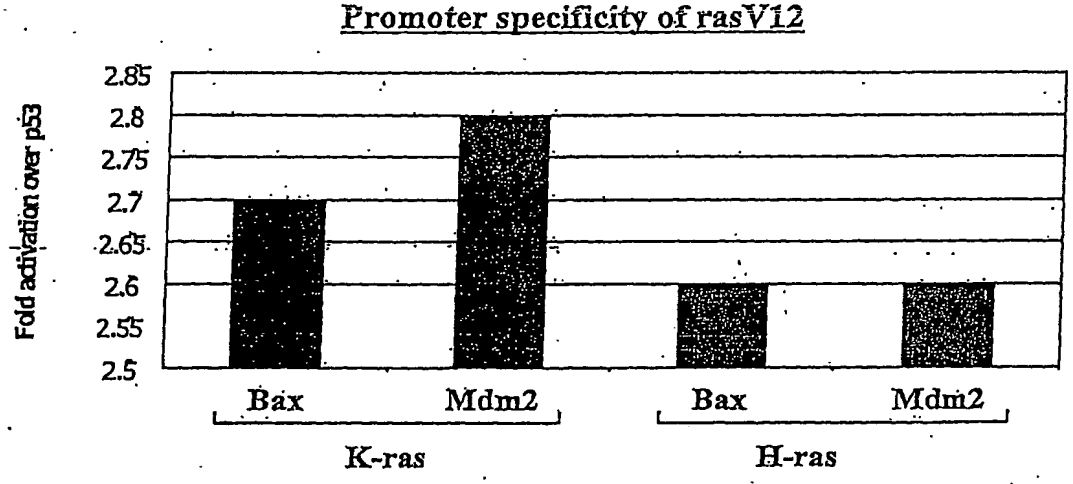


Figure 5A

H-rasV12 activates endogenous ASPP2 to transactivate bax reporter (U2OS cells)

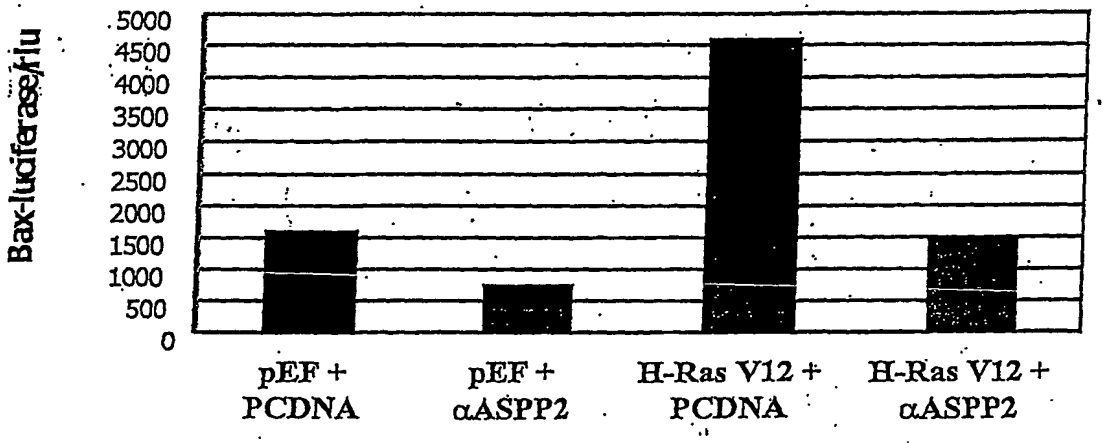
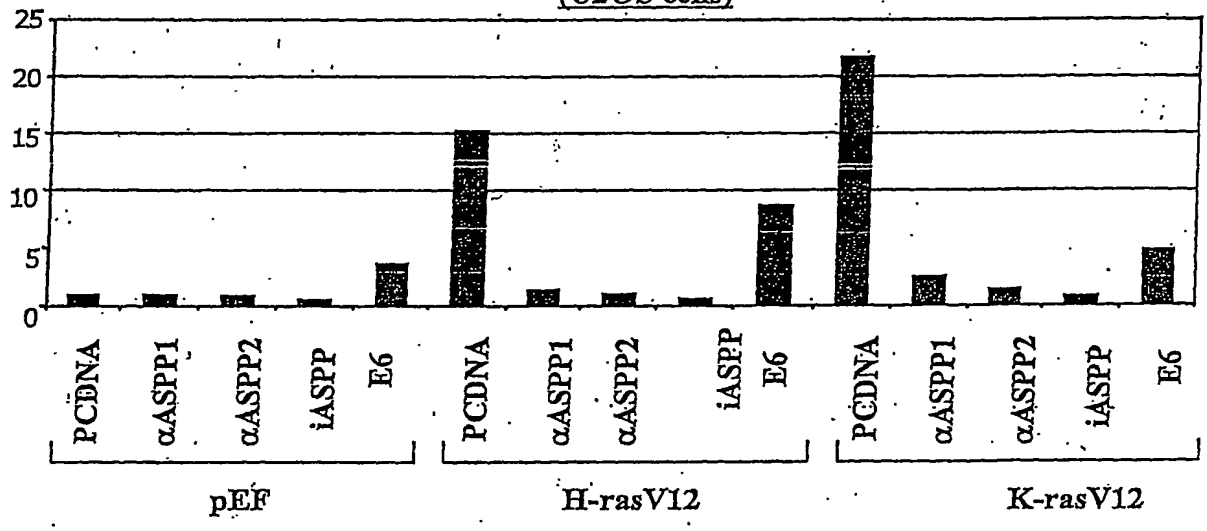


Figure 5B

H- and K-rasV12 activate endogenous ASPP1, ASPP2 and p53 to transactivate bax-reporter (U2OS cells)



Checking efficiency of Ras RNAi

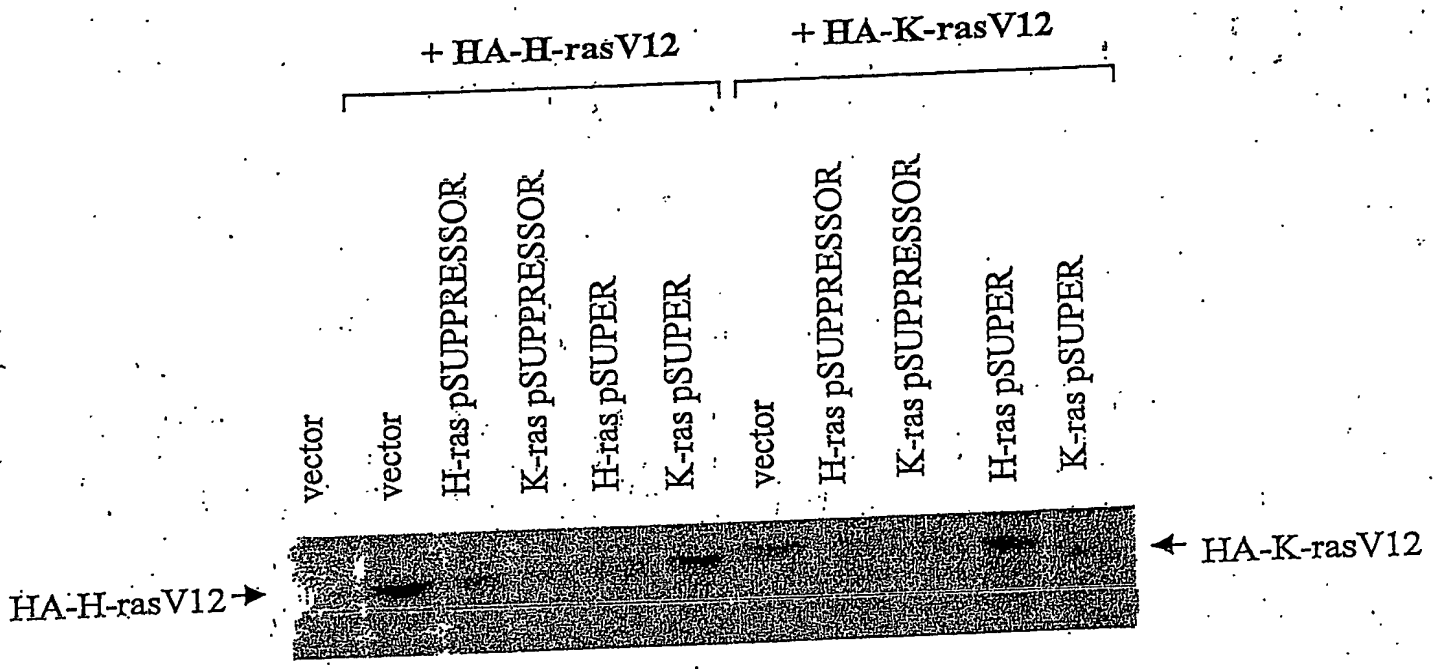


Fig 6A

Figure 6B

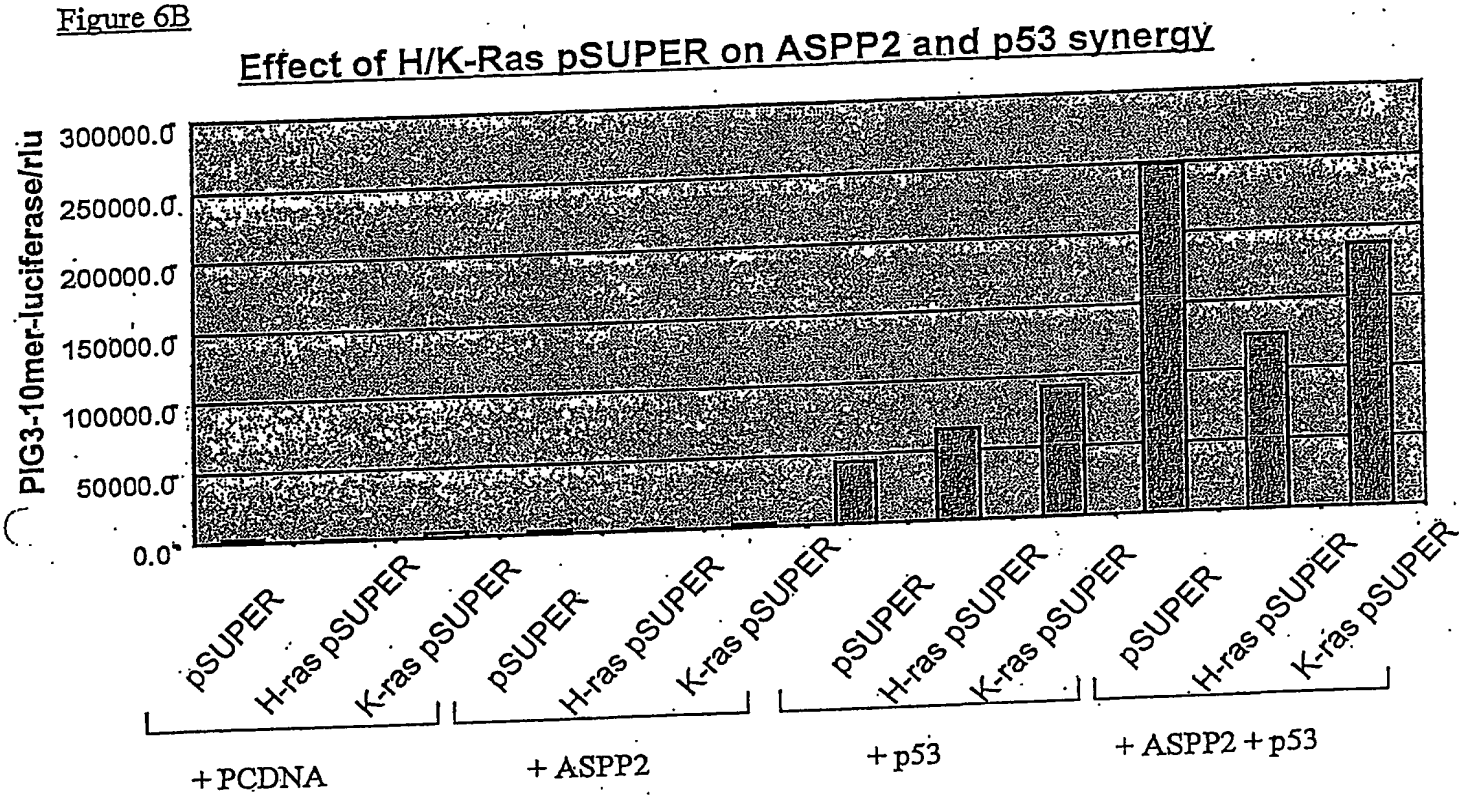
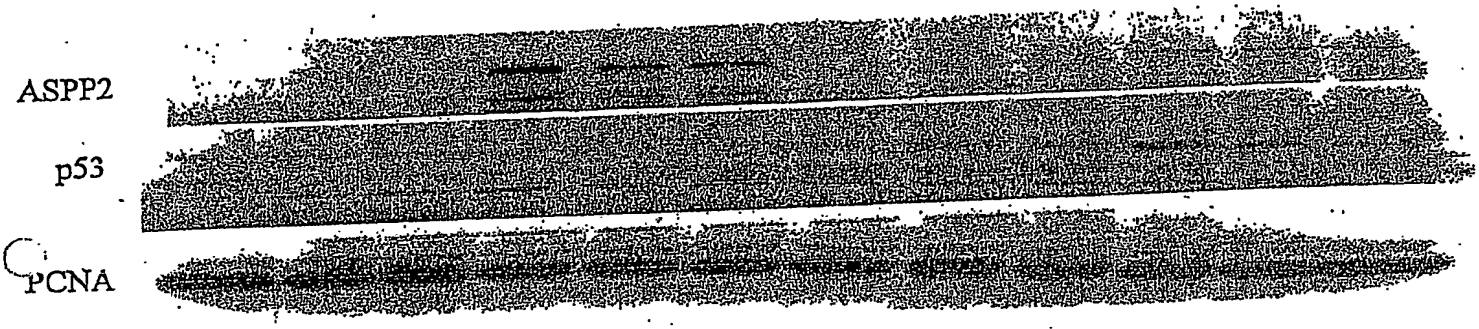


Figure 6C



Does Ras activate ASPP via its effector pathway?

Figure 7A: Effect of Ras and Raf CAAX: fold activation over P53 (Bax- reporter)

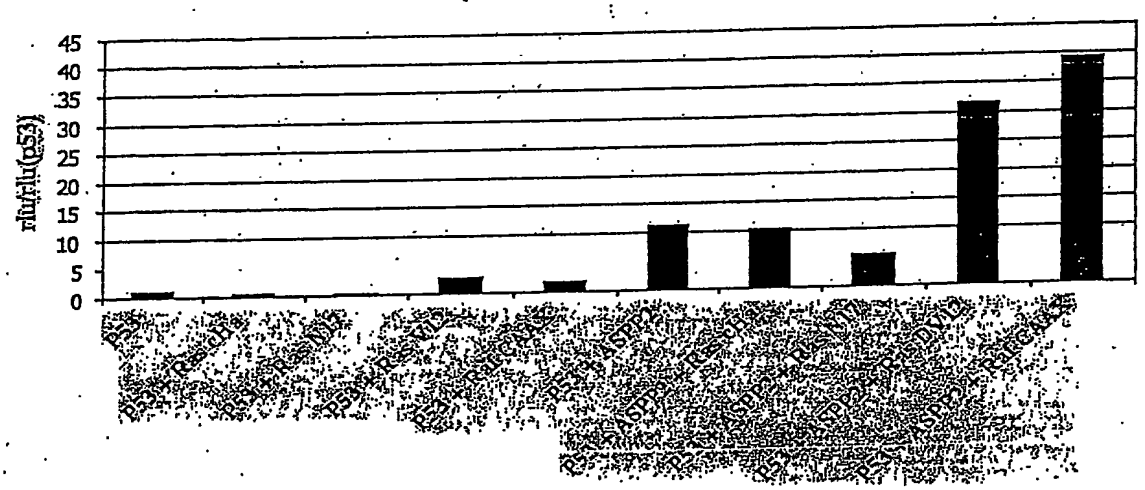


Figure 7B Effect of activated Raf CX on ASPP2 & p53 synergy

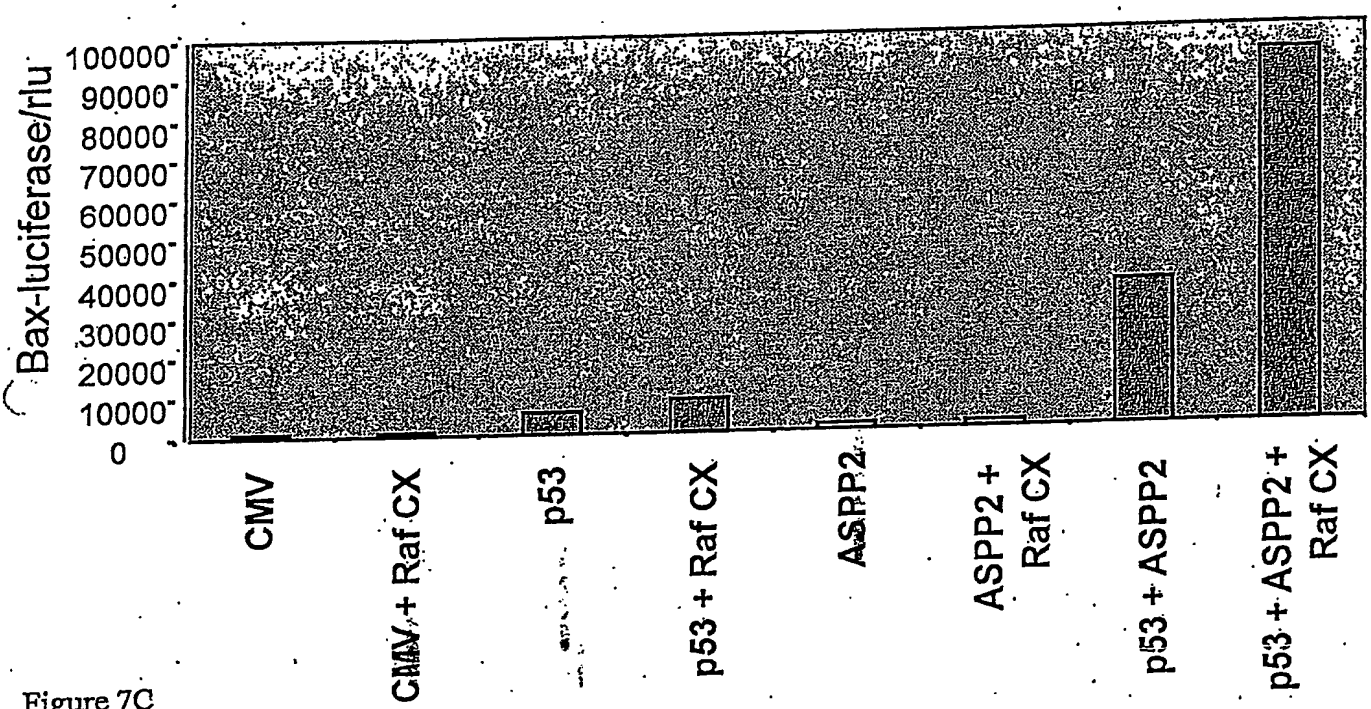
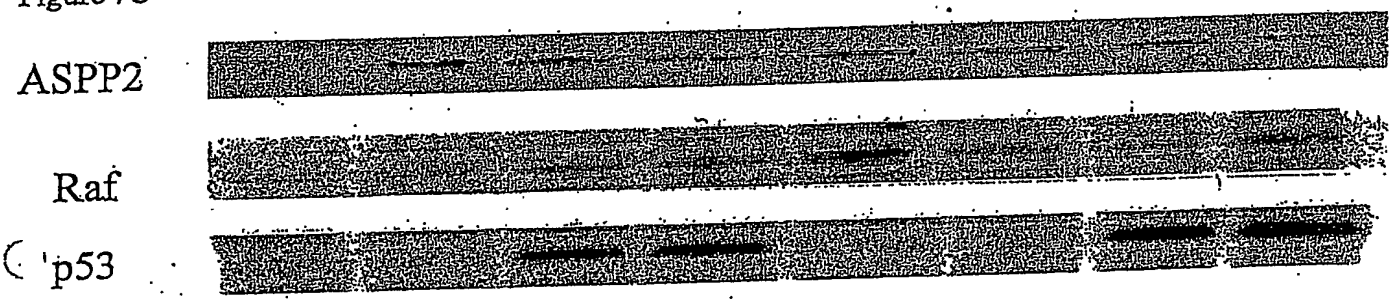


Figure 7C



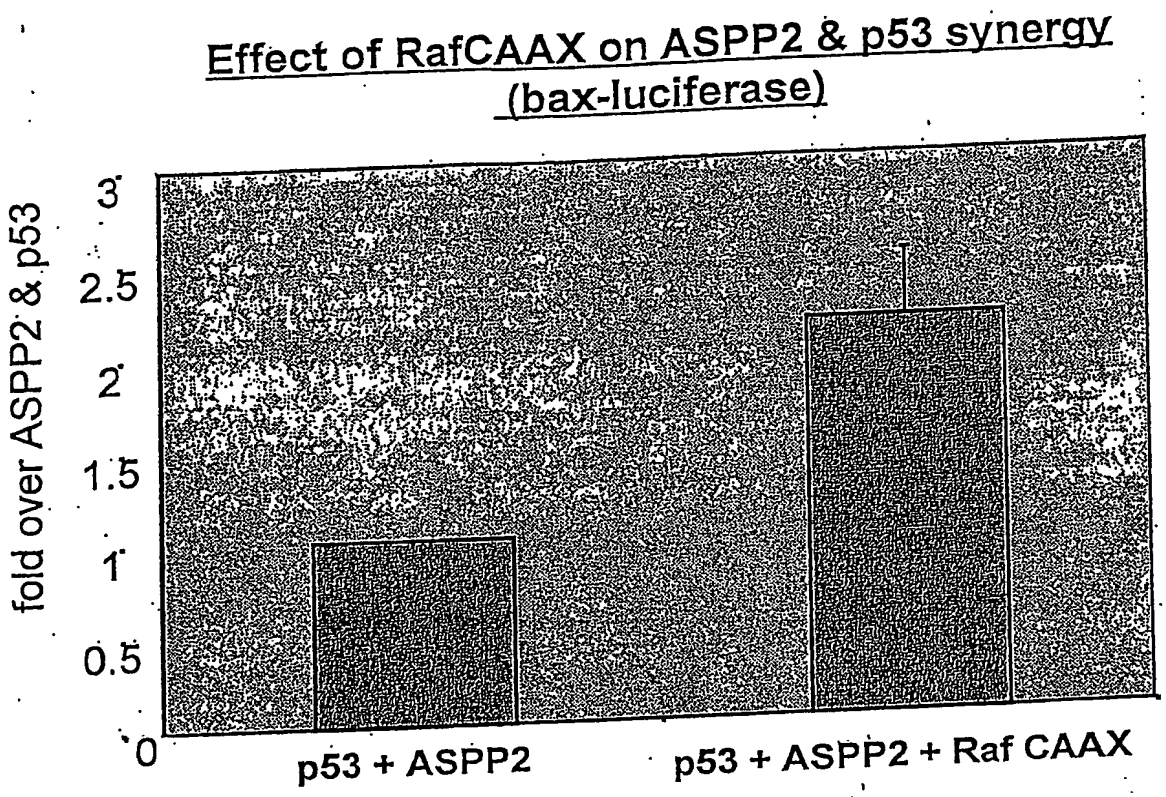

Effect of RafCAAX on ASPP2 & p53 synergy
(bax-luciferase)
fold over ASPP2 & p53
3
2.5
2
1.5
1
0.5
0
p53 + ASPP2
p53 + ASPP2 + Raf CAAX

Fig 7D

Figure 8A

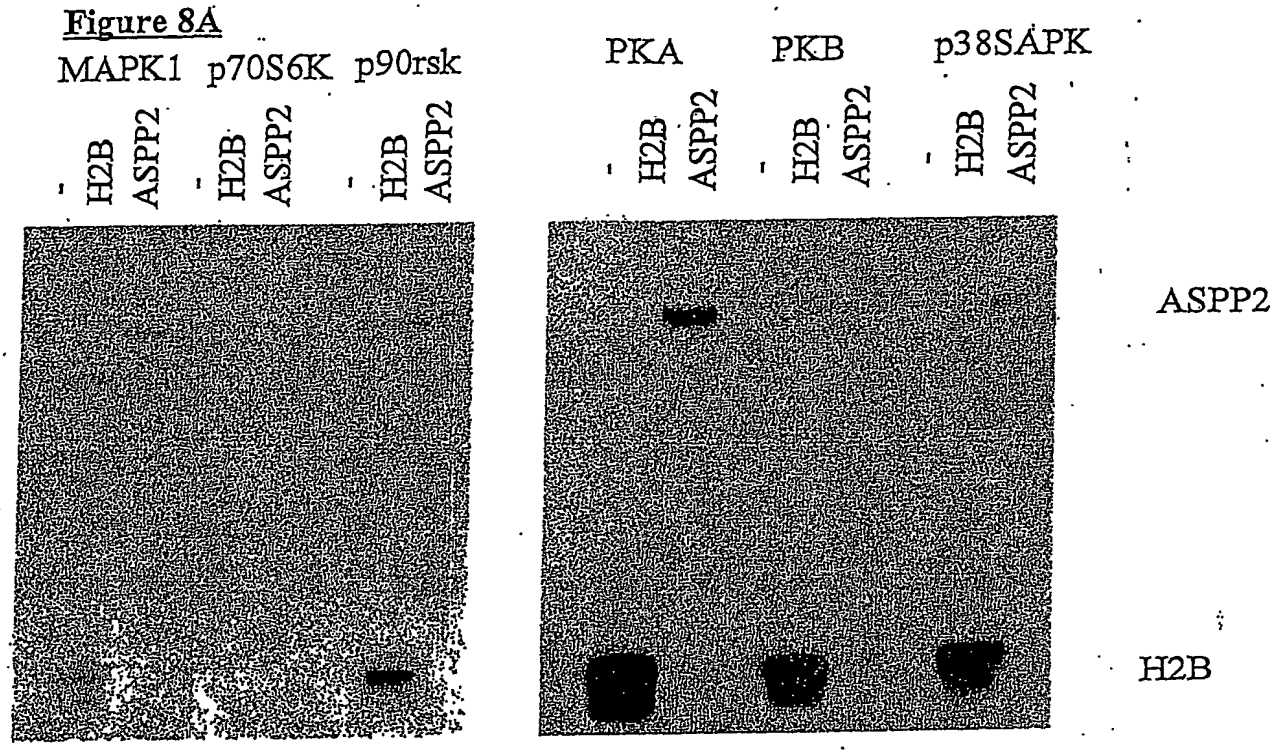


Figure 8B

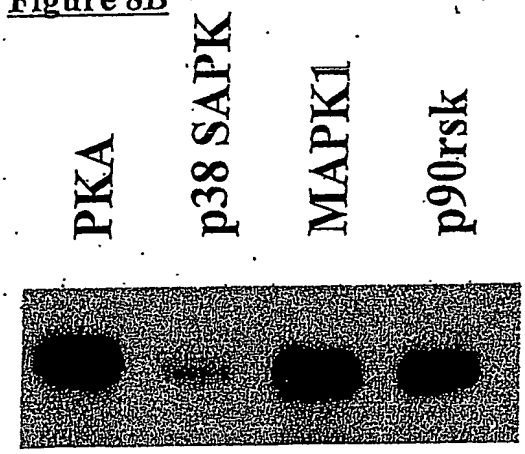


Figure 8C

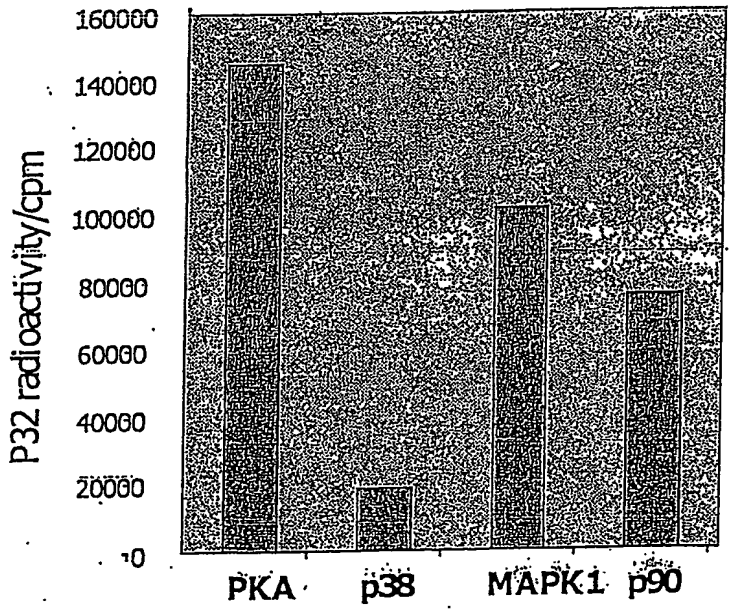
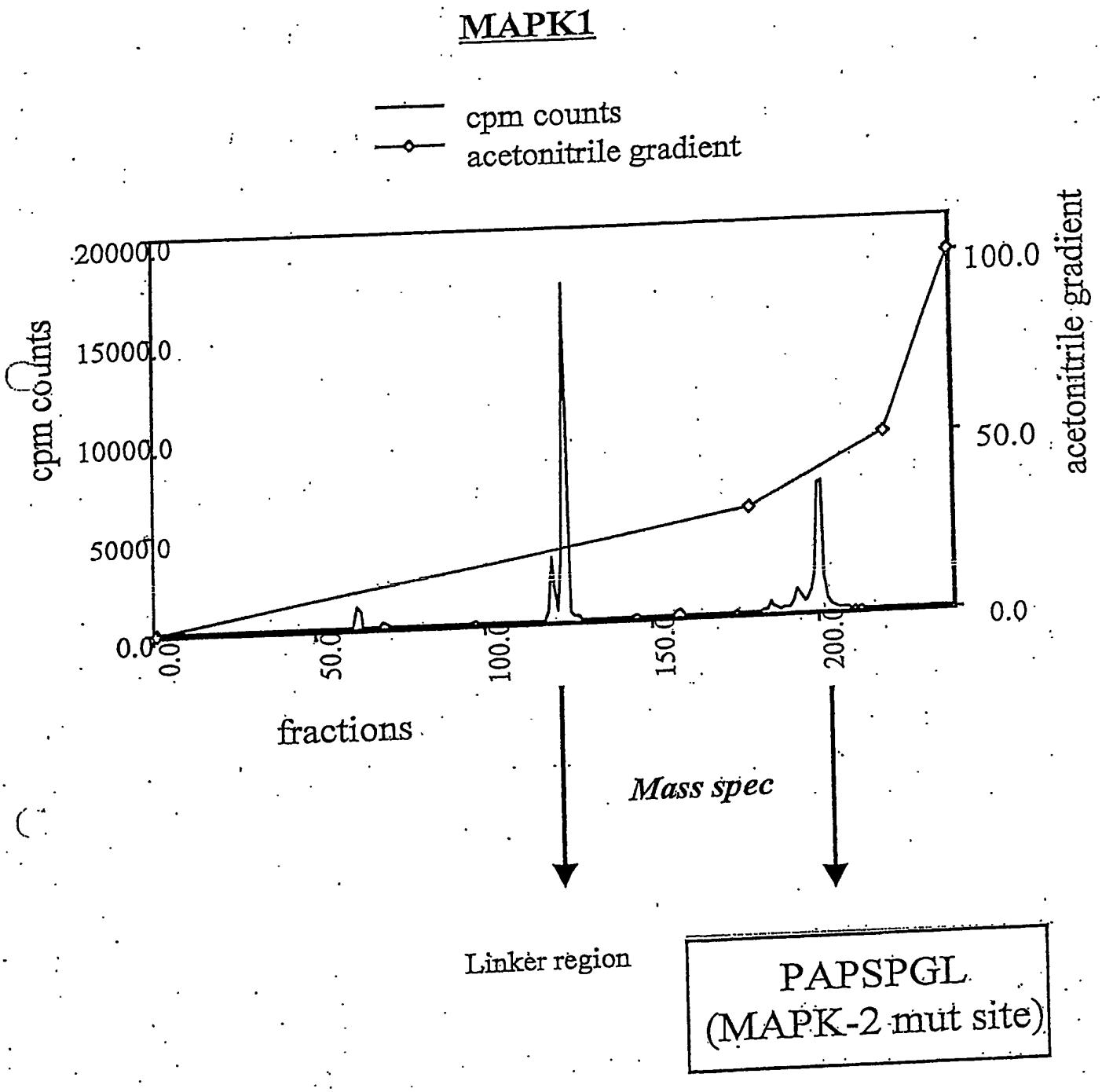


Figure 8D.


MAPK1
cpm counts
acetonitrile gradient
20000.0
15000.0
10000.0
5000.0
0.0
cpm counts
100.0
50.0
0.0
acetonitrile gradient
0.0
50.0
100.0
150.0
200.0
fractions
Mass spec
Linker region
PAPSPGL
(MAPK-2 mut site)

ASPP2 Mutants

C-term of ASPP2:

550 - QPRVLLSPSIPSVGQDQTLSPGSKQESPPAAAVRPFTPQPS
KDTLLPPFRKPQTVAASSIYSMYTQQQAPGKNFQQAVQS
ALTKTHTRGPHFSSVYGKPVIAAAQNQQQHPENIYSNSQ
GKPGSPEPETEPVSSVQENHENERIPRPLSPTKLLPFLSNP
YRNQSDADLEALRKKLSNAPRPLKKRSSITEPEGPNGPNI
QKLLYQRTTIAAMETSVPSYPSKSASVTASSESPVEIQNP
YLHVEPEKEVVSLVPESLSPEDVGNASTENSDMPAPSPGL
DYEPEGVPDNSPNLQNNPE - 849

S —— MAPK sites
SS —— PKA site

Fig 9

Figure 10A

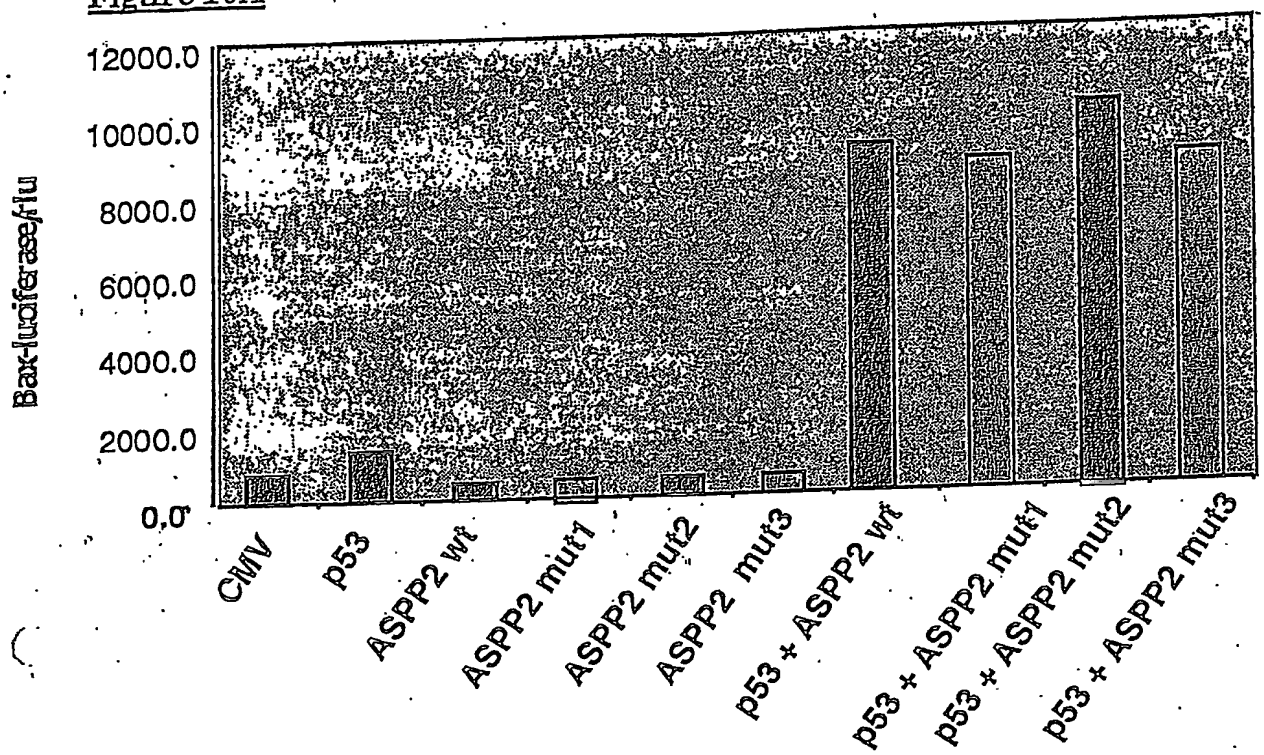


Figure 10B

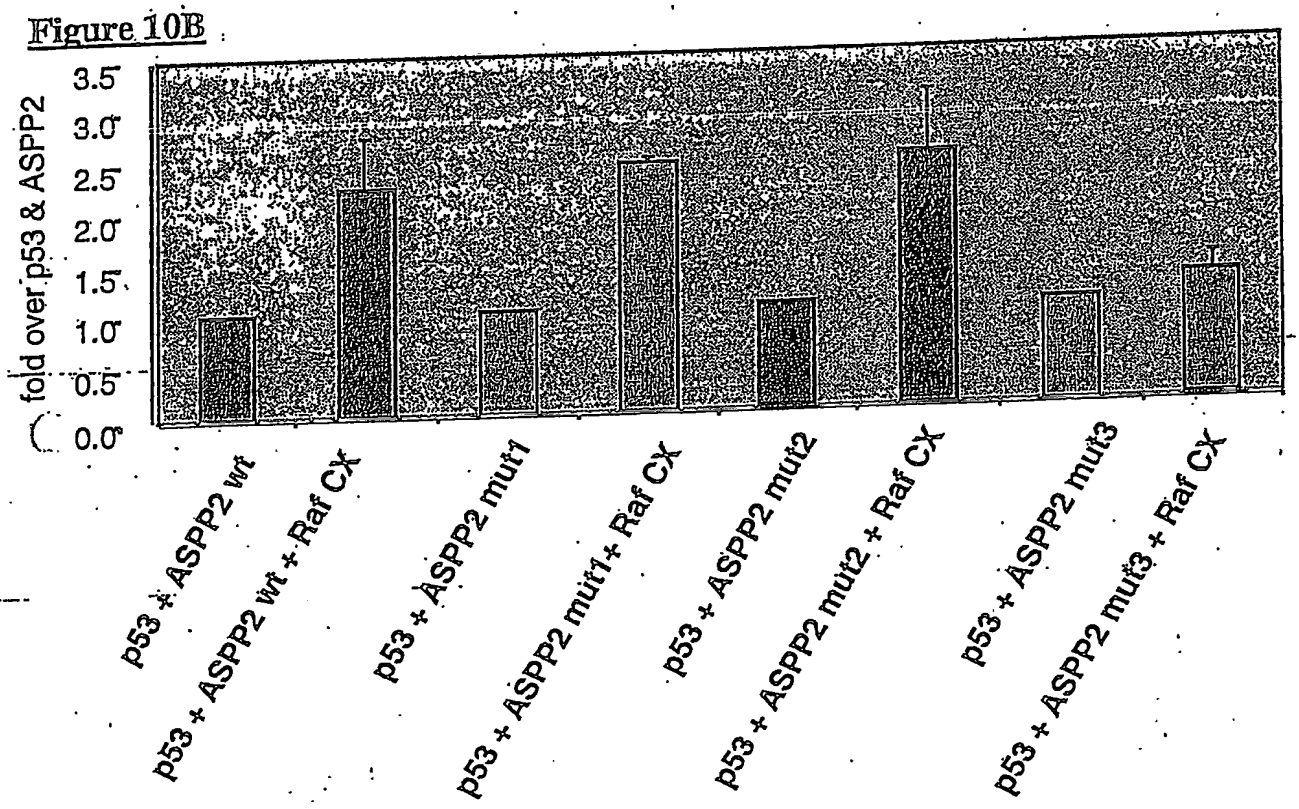


Figure 11A

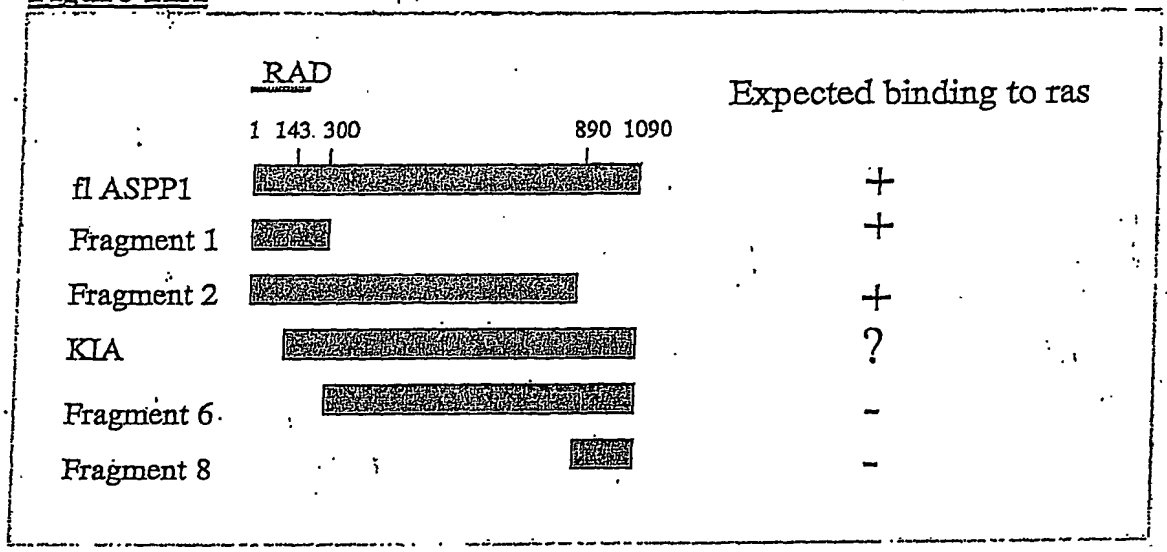
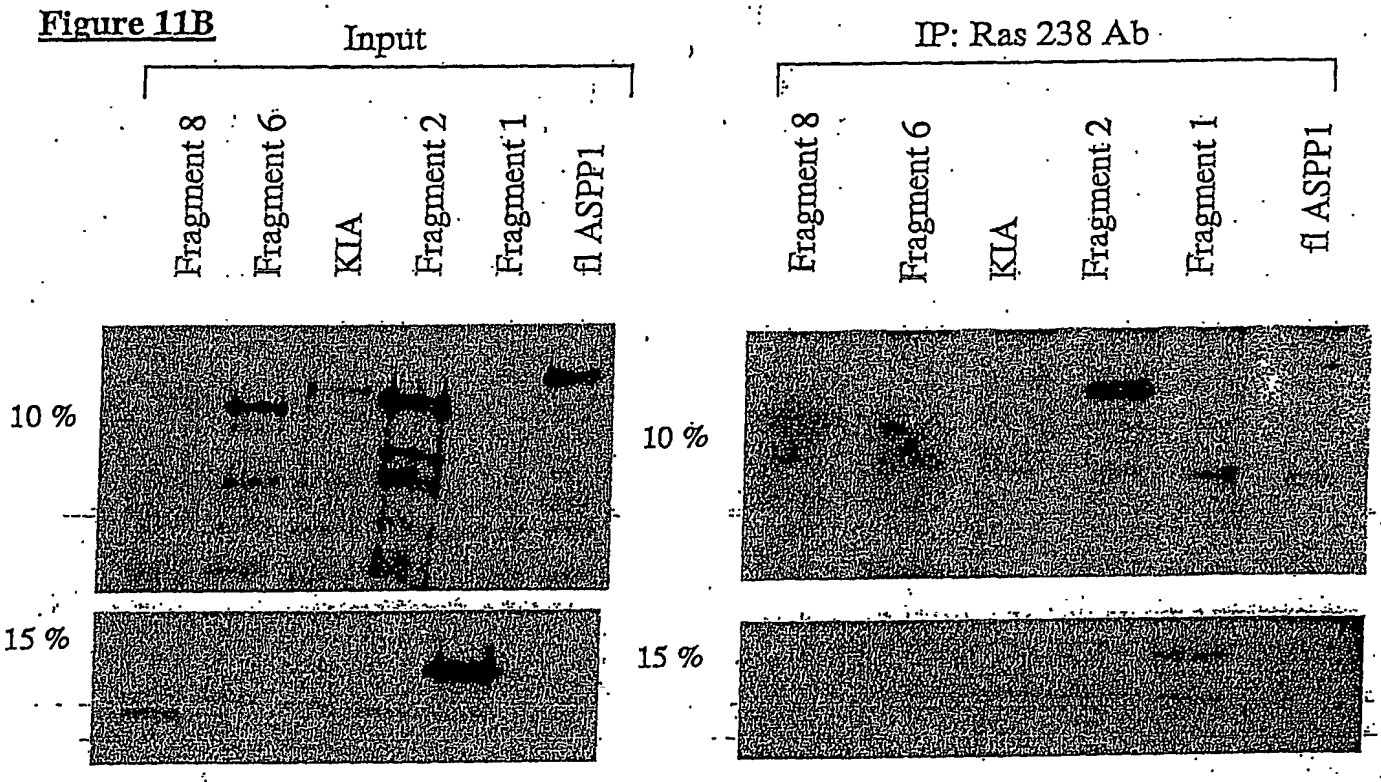


Figure 11B



Endogenous Ras binds ASPP2 after stimulation with EGF

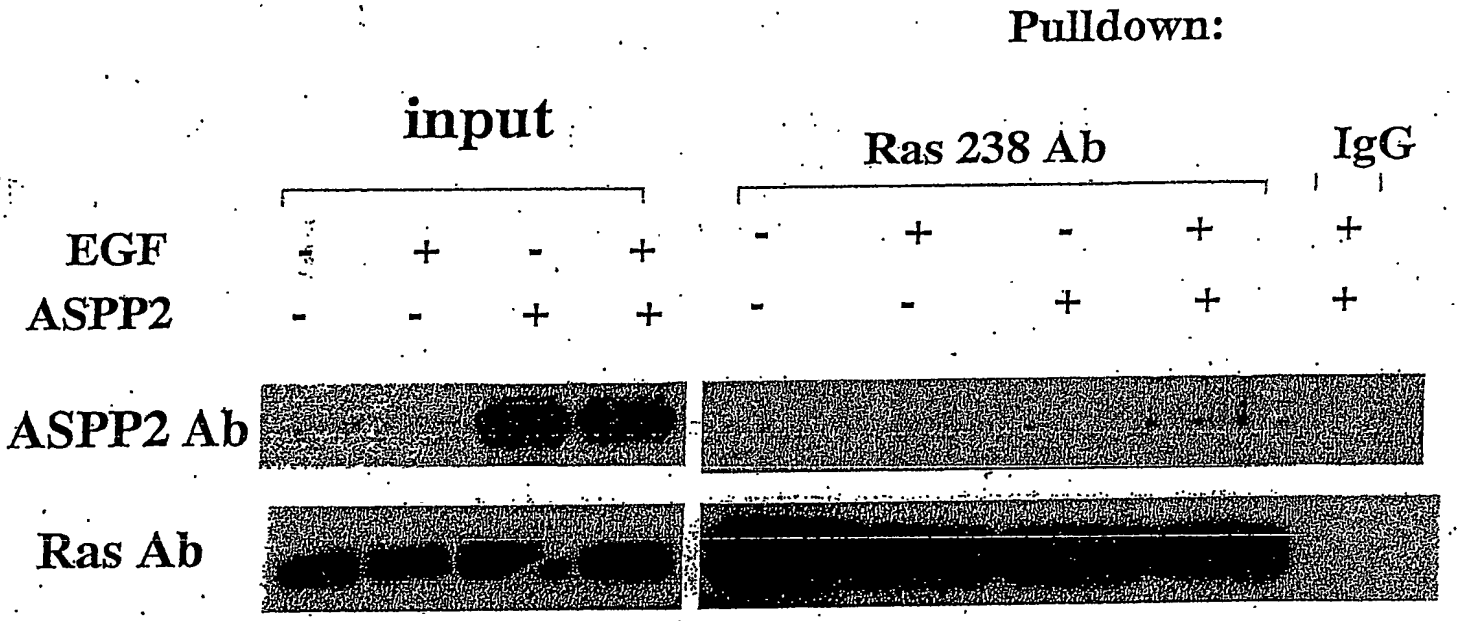


Fig 12

Endogenous ASPP1 and ASPP2 bind endognous Ras after EGF and 20% FCS stimulation

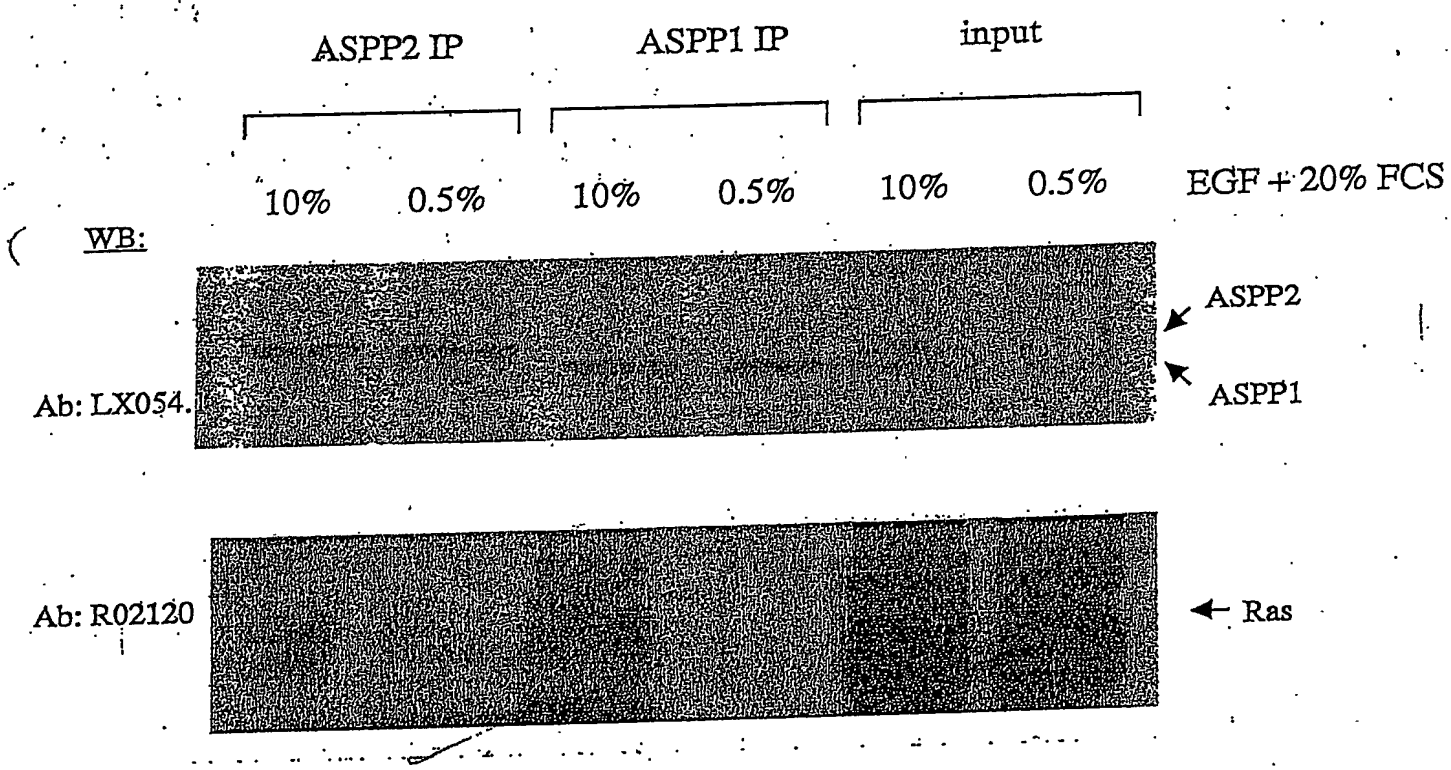


Figure 13

Figure 14A

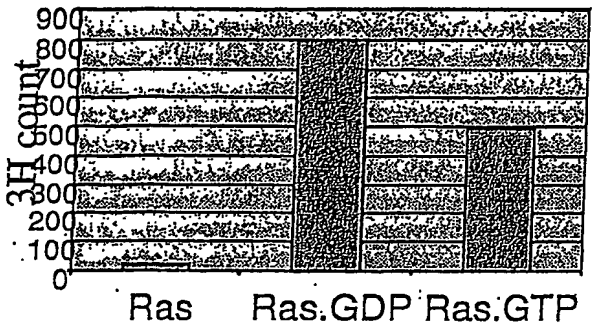


Figure 14B

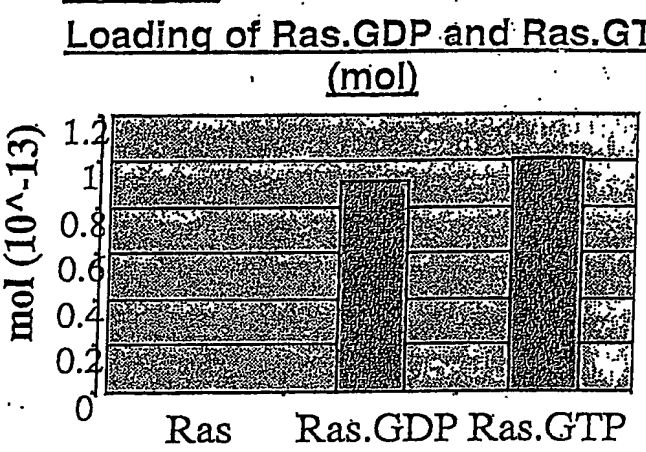


Figure 14C

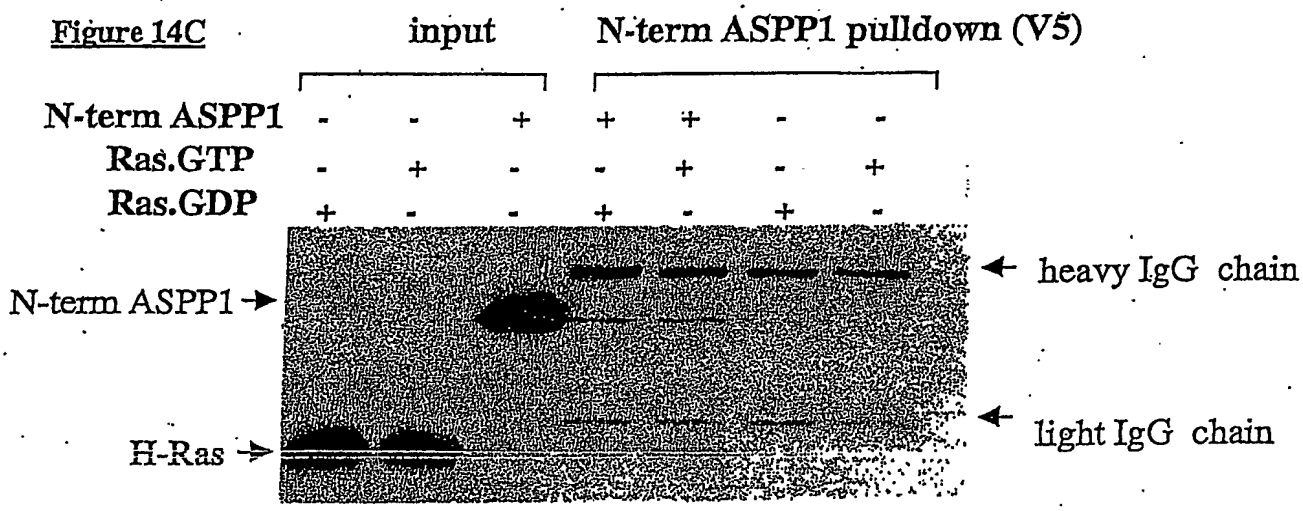
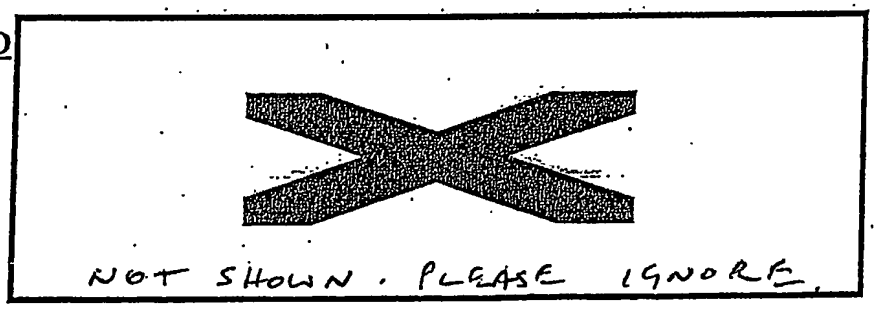


Figure 14D



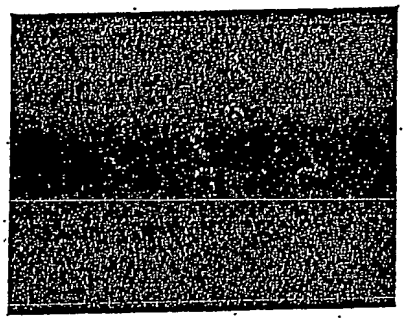
ASPP2 + H-RasV12

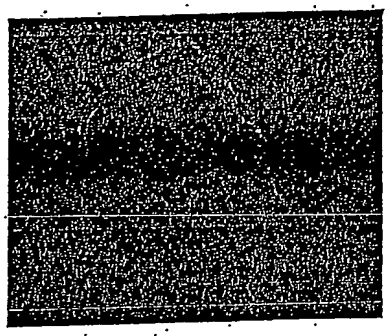
ASPP2 + H-RasV12

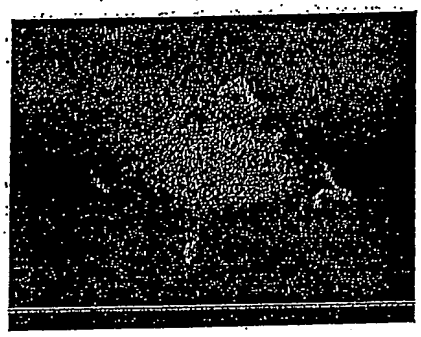
ASPP2

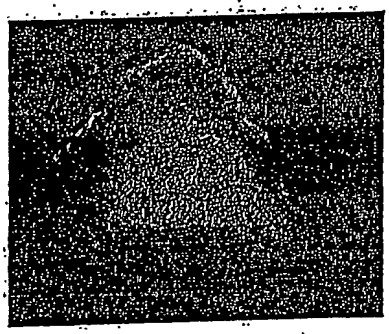
H-RasV12

Merged









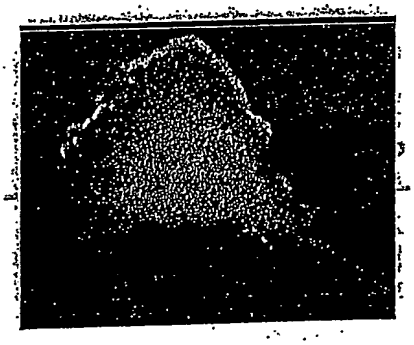


Fig 15

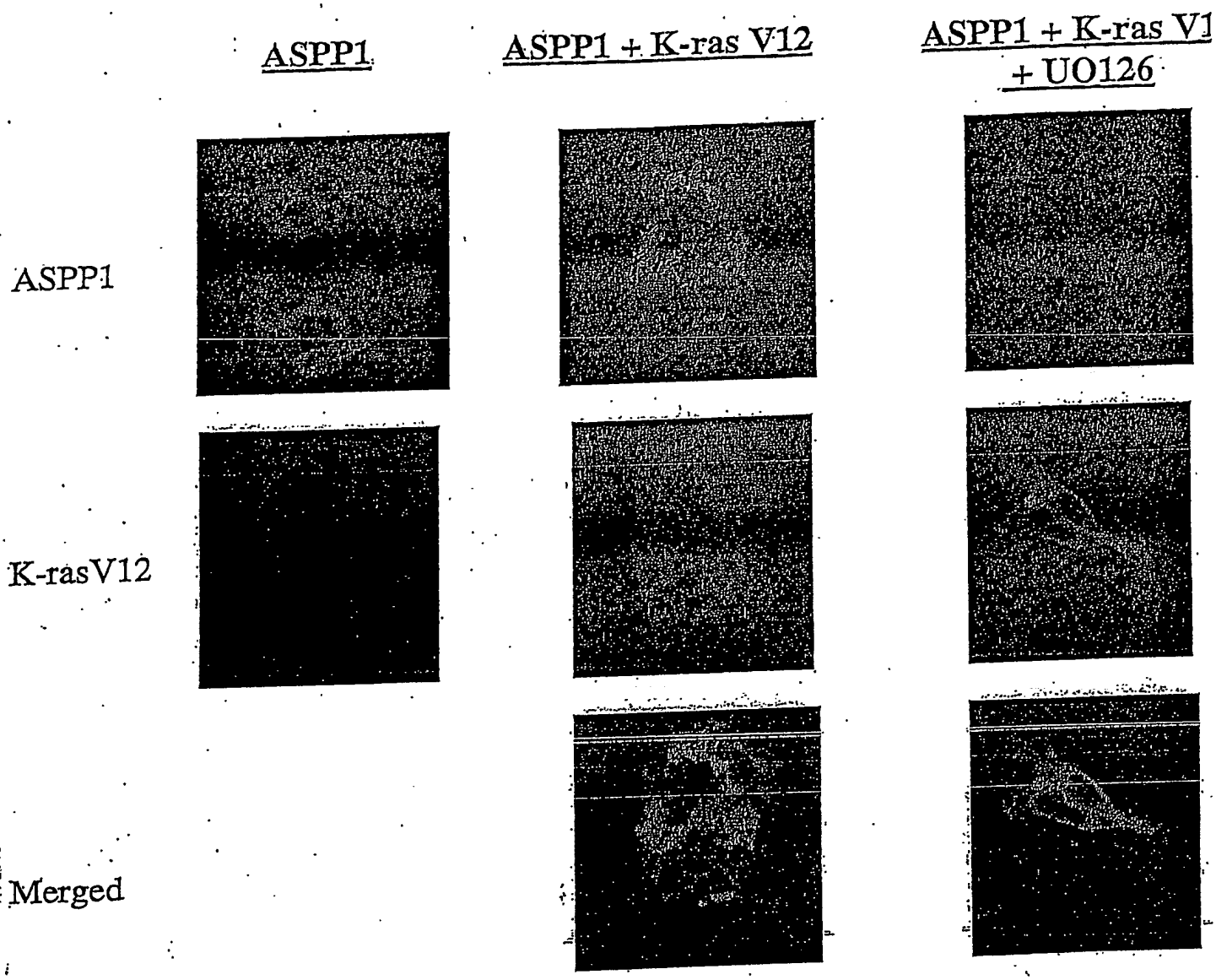

ASPP1
ASPP1 + K-ras V12
ASPP1 + K-ras V1
+ UO126
ASPP1
K-rasV12
Merged

Fig 16

Figure 17a

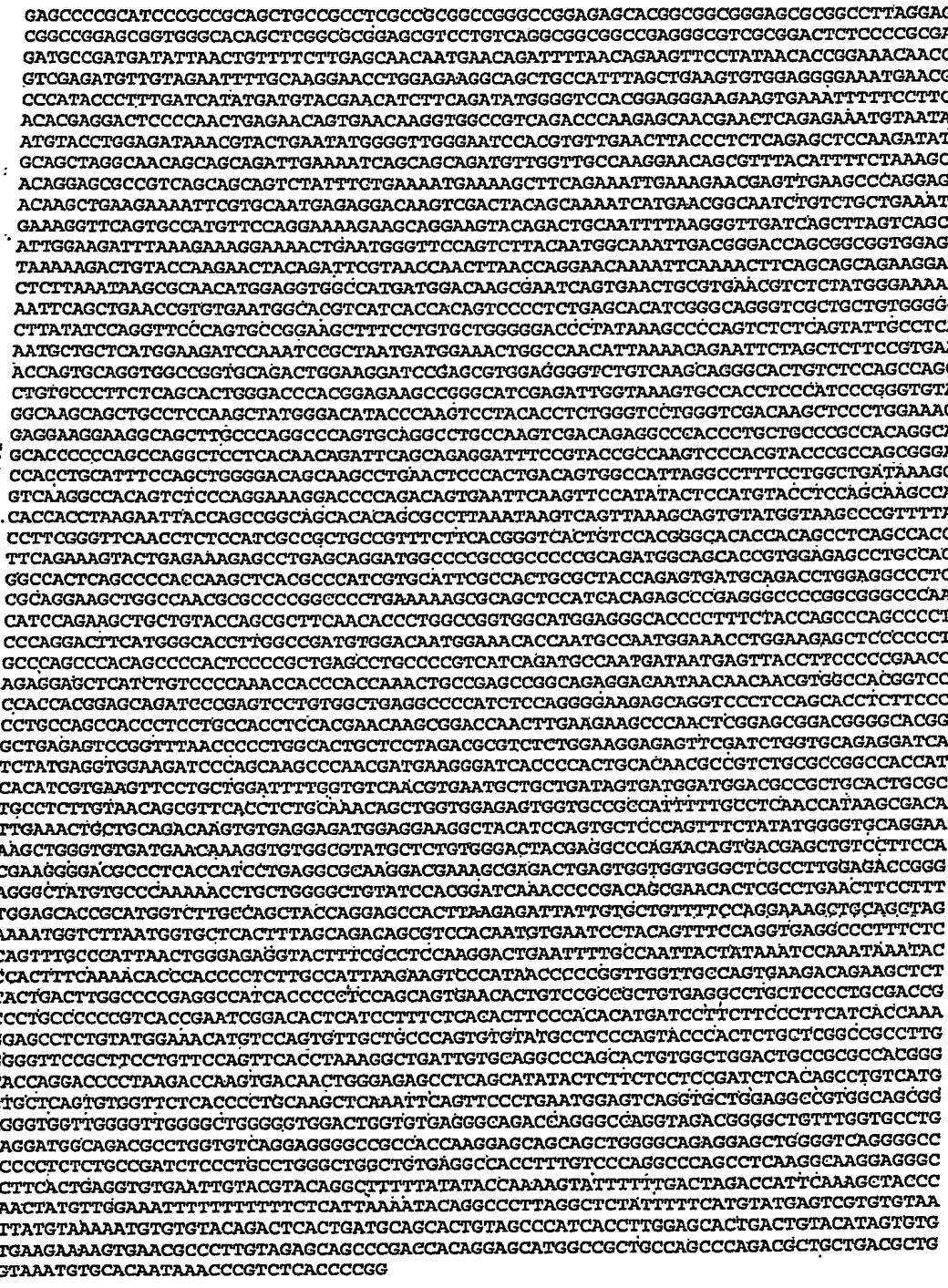

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Figure 17b

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GAAACTTGCTACAGACTTACCCGTAATATTTGTCAAGATCATAGCTGACTTTAAAAACAGTTGTAATAAACTTTTTGATG
CT

Figure 17c

MMPMILTVFLSNNEQILTEVPITPETTCRDVVEFCKEPGEGSCHLAEVWRGNERPIPFDHMMYEHLQIWGPRREEVKFFL
RHEDSPTENSEQGGRQTQEQRTQRNVINVPGDKRTEYGVGNPRVELTLSELQDMAARQQQQIENQQQMLVAKEQRLHFLK
QQERRQQQSISENEKLQKLKERVEAQENKLKKIRAMRGQVDYSKIMNGNLSAEIERFSAMFQEKKQEVQTAILRVDQLSQ
QLEDLKKGKLNGFQSYNGKLTGPAAVELKRLYQELQIRNQLNQEQNSKLQQQKELLNKRNMEVAMMDKRISELRERLYGK
KIQLNRVNGTSSPQSPLSTSGRVAAVGPYIQVPSAGSFPVLGDPIKPQSLSIASNAAHGRSKSANDGNWPTLKQNSSSSV
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NIQKLLYQRFNTLAGGMEGTPFYQPSPSQDFMGTLADVDNGNTNANGNLEELPPAQPTAPLPAEPAPSSDANDNELPSPE
PEELICPQTTHQTAEPAEDNNNNVATVPTTEQIPSPVAEAPSPGEEQVPPAPLPPASHPPATSTNKRTNLKKPNSERTGH
GLRVRFNPLALLLDASLEGEFDLVQRIIYEVEDPSKPNDEGITPLHNAVCAGHHHIVKFLLDFGVNVNAADSDGWTPLHC
AASCNSVHLCKQLVESGAAIFASTISDIETAADKCEEMEEGYIQCSQFLYGVQEKLGVMNKGVAYALWDYEAQNSDELSF
HEGDALTILRRKDESETEWWWARLGDREGYVPKNLLGLYPRIKPRQRTLA

Figure 17d

MMPMFLTVYLSNNEQHFTEVPVTPETICRDVVDLCKEPGESDCHLAEVWCGSERPVADNERMFDVLQRFGSQRNEVRFFL
RHERPPGRDIVSGPRSQDPSLKRNGVKVPGEYRRKENGVNSPRMDLTLAELQEMASRQQQQIEAQQQLLATKEQRLKFLK
QQDQRQQQQVAEQEKLKRLKEIAENQEAKLKKVRALKGHVEQKRLSNGKLVEEIEQMNNLFQQKQRELVLAVSKVEELTR
QLEMLKNGRIDSHHDNQSAVAELDRLYKELQLRNKLNQEQNAKLQQQRECLNKRNSEVAVMDKRVNELRDRLWKKKAALQ
QKENLPVSSDGNLPQQAASAPSRVAAVGPYIQSSTMPRMPSRPELLVKPALPDGSLVIQASEGPMKIQTLPNMRSGAASQ
TKGSKIHPVGPDWSPSNADLFPSQGSASVPQSTGNALDQVDDGEVPLREKEKKVRPFSMFDAVDQSNAPPSFGTLRKNQS
SEDILRDAQVANKNVAKVPPPVPTKPKQINLPYFGQTNQPPSDIKPDGSSQQLSTVVPSMGTKPKPAGQQPRVLLSPSIP
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FSSVYGKPVIAAAQNQQQHPENIYSNSQGKPGSPEPETEPVSSVQENHENERIPRPLSPTKLLPFLSNPYRNQSDADLEA
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VSLVPESLSPEDVGNASTENSDMPAPSPGLDYEPEGVPDNSPNLQNNPEEPNPEAPHVLDVYLEEYPPYPPPPYPSGEPE
GPGEDSVSMRPPEITGQVSLPPGKRTNLRKTGSERIAHGMRVKFNPLALLLDSSLEGEFDLVQRIIYEVDDPSLPNDEGI
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TQCSQFLYGVQEKMGIMNKGVIYALWDYEPQNDDELPMKEGDCMTIIHREDEDEIEWWWARLNDKEGYVPRNLLGLYPRI
KPRQRSLA

H-ras wild type DNA

Figure 18a

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CCCACTATAGAGGATTCCTACCGGAAGCAGGTGGTCATTGATGGGGAGAC
GTGCCTGTTGGACATCCTGGATACCGCCGGCCAGGAGGAGTACAGCGCCA
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CAAACGGGTGAAGGACTCGGATGACGTGCCCATGGTGCTGGTGGGGAAC
AAGTGTGACCTGGCTGCACGCACTGTGGAATCTCGGCAGGCTCAGGACCT
CGCCCGAAGCTACGGCATCCCCTACATCGAGACCTCGGCCAAGACCCGGC
AGGGAGTGGAGGATGCCTTCTACACGTTGGTGCGTGAGATCCGGCAGCAC
AAGCTGCGGAAGCTGAACCCTCCTGATGAGAGTGGCCCCGGCTGCATGAG
CTGCAAGTGTGTGCTCTCCTGA

Figure 18b

MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCL
LDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHQYREQIKRVK
DSDDVPMVLVGNKCDLAARTVESRQAQDLARSYGIPYIETSAKTRQGVEDAF
YTLVREIRQHKLRKLNPPDESGPGCMSCKCVLS

H-ras oncogenic DNA

Figure 18c

ATGACGGAATATAAGCTGGTGGTGGTGGGCGCCGTCGGTGTGGGCAAGA
GTGCGCTGACCATCCAGCTGATCCAGAACCATTTTGTGGACGAATACGAC
CCCACTATAGAGGATTCCTACCGGAAGCAGGTGGTCATTGATGGGGAGAC
GTGCCTGTTGGACATCCTGGATACCGCCGGCCAGGAGGAGTACAGCGCCA
TGCGGGACCAGTACATGCGCACCGGGGAGGGCTTCCTGTGTGTGTTTGCC
ATCAACAACACCAAGTCTTTTGAGGACATCCACCAGTACAGGGAGCAGAT
CAAACGGGTGAAGGACTCGGATGACGTGCCCATGGTGCTGGTGGGGAAC
AAGTGTGACCTGGCTGCACGCACTGTGGAATCTCGGCAGGCTCAGGACCT
CGCCCGAAGCTACGGCATCCCCTACATCGAGACCTCGGCCAAGACCCGGC
AGGGAGTGGAGGATGCCTTCTACACGTTGGTGCGTGAGATCCGGCAGCAC
AAGCTGCGGAAGCTGAACCCTCCTGATGAGAGTGGCCCCGGCTGCATGAG
CTGCAAGTGTGTGCTCTCCTGA

Figure 18d

MTEYKLVVVGAVGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCL
LDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHQYREQIKRVK
DSDDVPMVLVGNKCDLAARTVESRQAQDLARSYGIPYIETSAKTRQGVEDAF
YTLVREIRQHKLRKLNPPDESGPGCMSCKCVLS-

K ras 4B wild type DNA

Figure 18e

ATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAG
TGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCC
AACAATAGAGGATTCCTACAGGAAGCAAGTAGTAATTGATGGAGAAACC
TGTCTCTTGGATATTCTCGACACAGCAGGTCAAGAGGAGTACAGTGCAAT
GAGGGACCAGTACATGAGGACTGGGGAGGGCTTTCTTTGTGTATTTGCCA
TAAATAATACTAAATCATTTGAAGATATTCACCATTATAGAGAACAAATT
AAAAGAGTTAAGGACTCTGAAGATGTACCTATGGTCCTAGTAGGAAATAA
ATGTGATTTGCCTTCTAGAACAGTAGACACAAAACAGGCTCAGGACTTAG
CAAGAAGTTATGGAATTCCTTTTATTGAAACATCAGCAAAGACAAGACAG
GGTGTTGATGATGCCTTCTATACATTAGTTCGAGAAATTCGAAAACATAA
AGAAAAGATGAGCAAAGATGGTAAAAAGAAGAAAAAGAAGTCAAAGAC
AAAGTGTGTAATTATGTAA

Figure 18f

MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCL
LDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHHYREQIKRVK
DSEDVPMVLVGNKCDLPSRTVDTKQAQDLARSYGIPFIETSAKTRQGVDDAF
YTLVREIRKHKEKMSKDGKKKKKKSKTKCVIM-

K-ras 4B oncogenic DNA

Figure 18g

ATGACTGAATATAAACTTGTGGTAGTTGGAGCTGTCGGCGTAGGCAAGAG
TGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCC
AACAATAGAGGATTCCTACAGGAAGCAAGTAGTAATTGATGGAGAAACC
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Figure 18h

MTEYKLVVVGAVGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCL
LDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTKSFEDIHHYREQIKRVK
DSEDVPMVLVGNKCDLPSRTVDTKQAQDLARSYGIPFIETSAKTRQGVDDAF
YTLVREIRKHKEKMSKDGKKKKKKSKTKCVIM-

Figure 19a

```
atggcggcgg cggcggcggc gggcgcgggc ccggagatgg tccgcgggca ggtgttcgac
gtggggccgc gctacaccaa cctctcgtac atcggcgagg gcgcctacgg catggtgtgc
tctgcttatg ataatgtcaa caaagttcga gtagctatca agaaaatcag ccccttgag
caccagacct actgccagag aaccctgagg gagataaaaa tcttactgcg cttcagacat
gagaacatca ttggaatcaa tgacattatt cgagcaccaa ccatcgagca aatgaaagat
gtatatatag tacaggacct catggaaaca gatctttaca agctcttgaa gacacaacac
ctcagcaatg accatatctg ctatttttctc taccagatcc tcagagggtt aaaatatatc
cattcagcta acgttctgca ccgtgacctc aagccttcca acctgctgct caacaccacc
tgtgatctca agatctgtga ctttggcctg gcccgtgttg cagatccaga ccatgatcac
acagggttcc tgacagaata tgtggccaca cgttggtaca gggctccaga aattatgttg
aattccaagg gctacaccaa gtccattgat atttggtctg taggctgcat tctggcagaa
atgctttcta acaggcccat ctttccaggg aagcattatc ttgaccagct gaaacacatt
ttgggtattc ttggatcccc atcacaagaa gacctgaatt gtataataaa tttaaaagct
aggaactatt tgctttctct tccacacaaa aataaggtgc catggaacag gctgttccca
aatgctgact ccaaagctct ggacttattg gacaaaatgt tgacattcaa cccacacaag
aggattgaag tagaacaggc tctggcccac ccatatctgg agcagtatta cgacccgagt
gacgagccca tcgccgaagc accattcaag ttcgacatgg aattggatga cttgcctaag
gaaaagctca aagaactaat ttttgaagag actgctagat tccagccagg atacagatct
taa
```

Figure 19b

MAAAAAAGAGPEMVRGQVFDVGPRYTNLSYIGEGAYGMVCSAYD
NVNKVRVAIKKISPFEHQTYCQRTLREIKILLRFRHENIIGINDIIRAPTIEQMKDVY
IVQDLMETDLYKLLKTQHLSNDHICYFLYQILRGLKYIHSANVLHRDLKPSNLLL
NTTCDLKICDFGLARVADPDHDHTGFLTEYVATRWYRAPEIMLNSKGYTKSIDI
WSVGCILAEMLSNRPIFPGKHYLDQLKHILGILGSPSQEDLNCIINLKARNYLLSLP
HKNKVPWNRLFPNADSKALDLLDKMLTFNPHKRIEVEQALAHPYLEQYYDPSD
EPIAEAPFKFDMELDDLPKEKLKELIFEETARFQPGYRS

Figure 20a

```
   1 tcgggctgag gttcccgggc gggcgggcgc ggagagacgc gggaagcagg ggctgggcgg
  61 gggtcgcggc gccgcagcta gcgcagccag cccgagggcc gccgccgccg ccgcccagcg
 121 cgctccgggg ccgccggccg cagccagcac ccgccgcgcc gcagctccgg gaccggcccc
 181 ggccgccgcc gccgcgatgg gcaacgccgc cgccgccaag aagggcagcg agcaggagag
 241 cgtgaaagaa ttcttagcca aagccaaaga agattttctt aaaaaatggg aaagtcccgc
 301 tcagaacaca gcccacttgg atcagtttga acgaatcaag accctcggca cgggctcctt
 361 cgggcgggtg atgctggtga aacacaagga gaccgggaac cactatgcca tgaagatcct
 421 cgacaaacag aaggtggtga aactgaaaca gatcgaacac accctgaatg aaaagcgcat
 481 cctgcaagct gtcaactttc cgttcctcgt caaactcgag ttctccttca aggacaactc
 541 aaacttatac atggtcatgg agtacgtgcc cggcggggag atgttctcac acctacggcg
 601 gatcggaagg ttcagtgagc cccatgcccg tttctacgcg gcccagatcg tcctgacctt
 661 tgagtatctg cactcgctgg atctcatcta cagggacctg aagccggaga atctgctcat
 721 tgaccagcag ggctacattc aggtgacaga cttcggtttc gccaagcgcg tgaagggccg
 781 cacttggacc ttgtgcggca cccctgagta cctggccccc gagattatcc tgagcaaagg
 841 ctacaacaag gccgtggact ggtgggccct gggggttctt atctatgaaa tggccgctgg
 901 ctacccgccc ttcttcgcag accagcccat ccagatctat gagaagatcg tctctgggaa
 961 ggtgcgcttc ccttcccact tcagctctga cttgaaggac ctgctgcgga acctcctgca
1021 ggtagatctc accaagcgct ttgggaacct caagaatggg gtcaacgata tcaagaacca
1081 caagtggttt gccacaactg actggattgc catctaccag aggaaggtgg aagctccctt
1141 cataccaaag tttaaaggcc ctggggatac gagtaacttt gacgactatg aggaagaaga
1201 aatccgggtc tccatcaatg agaagtgtgg caaggagttt tctgagtttt aggggcatgc
1261 ctgtgccccc atgggttttc ttttttcttt tttctttttt ttggtcgggg gggtgggagg
1321 gttggattga acagccagag ggccccagag ttccttgcat ctaatttcac ccccacccca
1381 ccctccaggg ttaggggggag caggaagccc agataatcag agggacagaa acaccagctg
1441 ctcccccctca tcccccttcac cctcctgccc cctctcccac ttttcccttc ctctttcccc
1501 acagcccccc agcccctcag ccctcccagc ccacttctgc ctgttttaaa cgagtttctc
1561 aactccagtc agaccaggtc ttgctggtgt atccagggac agggtatgga aagaggggct
1621 cacgcttaac tccagccccc acccacaccc ccatcccacc caaccacagg ccccacttgc
1681 taagggcaaa tgaacgaagc gccaaccttc ctttcggagt aatcctgcct gggaaggaga
1741 gatttttagt gacatgttca gtgggttgct tgctagaatt tttttaaaaa aacaacaatt
1801 taaaatctta tttaagttcc accagtgcct ccctccctcc ttcctctact cccacccctc
1861 ccatgtcccc ccattcctca aatccatttt aaagagaagc agactgactt tggaaaggga
1921 ggcgctgggg tttgaacctc cccgctgcta atctcccctg ggcccctccc cggggaatcc
1981 tctctgccaa tcctgcgagg gtctaggccc ctttaggaag cctccgctct ctttttcccc
2041 aacagacctg tcttcaccct tgggctttga aagccagaca aagcagctgc ccctcttcct
2101 gccaaagagg agtcatcccc caaaaagaca gaggggagc cccaagccca agtctttcct
2161 cccagcagcg tttccccccca actccttaat tttattctcc gctagatttt aacgtccagc
2221 cttccctcag ctgagtgggg agggcatccc tgcaaaaggg aacagaagag gccaagtccc
2281 cccaagccac ggcccggggt tcaaggctag agctgctggg gaggggctgc ctgttttact
2341 cacccaccag cttccgcctc cacccatcctg ggcgcccctc ctccagctta gctgtcagct
2401 gtccatcacc tctcccccac tttctcattt gtgcttttttt ctctcgtaat agaaaagtgg
2461 ggagccgctg gggagccacc ccattcatcc ccgtatttcc cccctctcata acttctcccc
2521 atcccaggag gagttctcag gcctggggtg gggccccggg tgggtgcggg ggcgattcaa
2581 cctgtgtgct gcgaaggacg agacttcctc ttgaacagtg tgctgttgta aacatatttg
2641 aaaactatta ccaataaagt tttgtttaaa aaaaaaaaaa aaaaa
```

Figure 20b

```
MGNAAAAKKGSEQESVKEFLAKAKEDFLKKWESPAQNTAHLDQF
ERIKTLGTGSFGRVMLVKHKETGNHYAMKILDKQKVVKLKQIEHTLNEKRILQAVNFP
FLVKLEFSFKDNSNLYMVMEYVPGGEMFSHLRRIGRFSEPHARFYAAQIVLTFEYLHS
LDLIYRDLKPENLLIDQQGYIQVTDFGFAKRVKGRTWTLCGTPEYLAPEIILSKGYNK
AVDWWALGVLIYEMAAGYPPFFADQPIQIYEKIVSGKVRFPSHFSSDLKDLLRNLLQV
DLTKRFGNLKNGVNDIKNHKWFATTDWIAIYQRKVEAPFIPKFKGPGDTSNFDDYEEE
EIRVSINEKCGKEFSEF
```

PP1 alpha DNA

Figure 21a

ATGTCCGACAGCGAGAAGCTCAACCTGGACTCGATCATCGGGCGCCTGCT
GGAAGTGCAGGGCTCGCGGCCTGGCAAGAATGTACAGCTGACAGAGAAC
GAGATCCGCGGTCTGTGCCTGAAATCCCGGGAGATTTTTCTGAGCCAGCC
CATTCTTCTGGAGCTGGAGGCACCCCTCAAGATCTGCGGTGACATACACG
GCCAGTACTACGACCTTCTGCGACTATTTGAGTATGGCGGTTTCCCTCCCG
AGAGCAACTACCTCTTTCTGGGGGGACTATGTGGACAGGGGCAAGCAGTCC
TTGGAGACCATCTGCCTGCTGCTGGCCTATAAGATCAAGTACCCCGAGAA
CTTCTTCCTGCTCCGTGGGAACCACGAGTGTGCCAGCATCAACCGCATCTA
TGGTTTCTACGATGAGTGCAAGAGACGCTACAACATCAAACTGTGGAAAA
CCTTCACTGACTGCTTCAACTGCCTGCCCATCGCGGCCATAGTGGACGAA
AAGATCTTCTGCTGCCACGGAGGCCTGTCCCCGGACCTGCAGTCTATGGA
GCAGATTCGGCGGATCATGCGGCCCACAGATGTGCCTGACCAGGGCCTGC
TGTGTGACCTGCTGTGGTCTGACCCTGACAAGGACGTGCAGGGCTGGGGC
GAGAACGACCGTGGCGTCTCTTTTACCTTTGGAGCCGAGGTGGTGGCCAA
GTTCCTCCACAAGCACGACTTGGACCTCATCTGCCGAGCACACCAGGTGG
TAGAAGACGGCTACGAGTTCTTTGCCAAGCGGCAGCTGGTGACACTTTTC
TCAGCTCCCAACTACTGTGGCGAGTTTGACAATGCTGGCGCCATGATGAG
TGTGGACGAGACCCTCATGTGCTCTTTCCAGATCCTCAAGCCCGCCGACA
AGAACAAGGGGAAGTACGGGCAGTTCAGTGGCCTGAACCCTGGAGGCCG
ACCCATCACCCCACCCCGCAATTCCGCCAAAGCCAAGAAATAG

Figure 21b

MSDSEKLNLDSIIGRLLEVQGSRPGKNVQLTENEIRGLCLKSREIFLSQPILLEL
EAPLKICGDIHGQYYDLLRLFEYGGFPPESNYLFLGDYVDRGKQSLETICLLL
AYKIKYPENFFLLRGNHECASINRIYGFYDECKRRYNIKLWKTFTDCFNCLPIA
AIVDEKIFCCHGGLSPDLQSMEQIRRIMRPTDVPDQGLLCDLLWSDPDKDVQ
GWGENDRGVSFTFGAEVVAKFLHKHDLDLICRAHQVVEDGYEFFAKRQLVT
LFSAPNYCGEFDNAGAMMSVDETLMCSFQILKPADKNKGKYGQFSGLNPGG
RPITPPRNSAKAKK-

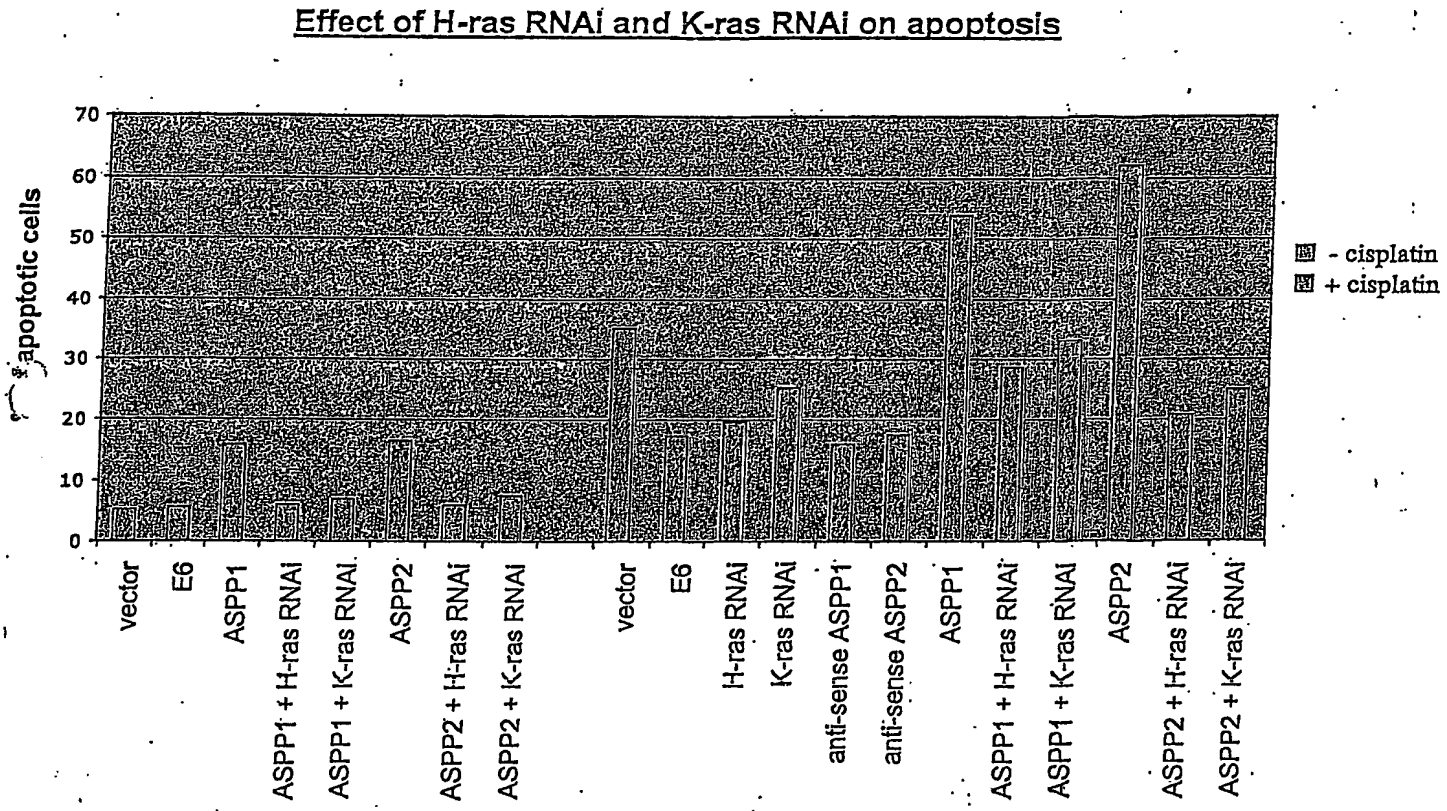
Effect of H-ras RNAi and K-ras RNAi on apoptosis
% apoptotic cells
70
60
50
40
30
20
10
0
vector
E6
ASPP1
ASPP1 + H-ras RNAi
ASPP1 + K-ras RNAi
ASPP2
ASPP2 + H-ras RNAi
ASPP2 + K-ras RNAi
vector
E6
H-ras RNAi
K-ras RNAi
anti-sense ASPP1
anti-sense ASPP2
ASPP1
ASPP1 + H-ras RNAi
ASPP1 + K-ras RNAi
ASPP2
ASPP2 + H-ras RNAi
ASPP2 + K-ras RNAi
- cisplatin
+ cisplatin

Figure 22